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Chitinase 3-like protein 1

for early diagnosis of acute kidney injury

Clinical validation in the human intensive care unit patient

Dissertation submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Chitinase 3-like protein 1 for early diagnosis of acute kidney injury: clinical validation in the human intensive care unit patient

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Dankzij mijn ouders

Voor pepe, meter en peter

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Abbreviation Key

4

4PL 4-parameter logistic, 84

A

ACE angiotensin-converting enzyme, 14
ACE2 angiotensin-converting enzyme 2, 14
ADP adenosine diphosphate, 18
ADQI Acute Dialysis Quality Initiative, 20
AKD acute kidney disease, 27
AKI acute kidney injury, 15
AKIN Acute Kidney Injury Network, 16
AKS acute kidney stress, 39
Ang II angiotensin II, 9
aPC activated protein C, 32
ARF acute renal failure, 15
AT1 type-1 Ang II, 14
AT2 type-2 Ang II, 14
ATP adenosine triphosphate, 18
AUC-ROC area under the receiver-operating characteristics curve, 63

B

BMI body mass index, 126

C

C creatine, 18
CAZy Carbohydrate-Active Enzymes database, 46
CCL2 C-C motif chemokine 2, 32
CDK cyclin-dependent kinase, 36
CDKI CDK inhibitor, 36
CHI3L1 chitinase 3-like protein 1, 40
CHI3L3 chitinase 3-like protein 3, 57
CHIA acidic mammalian chitinase, 57
CI confidence interval, 24
CK creatine kinase, 18
CKD chronic kidney disease, 21
CKD-EPI Chronic Kidney Disease Epidemiology Collaboration, 96
CL central laboratory, 163
CLP chitinase-like protein, 46
CO carbon monoxide, 43
CPB cardiopulmonary bypass, 118
cPC circulating protein C, 32
Cr creatinine, 9
CSA-AKI cardiac surgery-associated AKI, 117
CV coefficient of variation, 97

D

d day, 9
Da Dalton, 41
DAMP damage/danger-associated molecular pattern, 30
DM diabetes mellitus, 126
DMT-1 divalent metal transporter 1, 42

E

ECC extracorporeal circulation, 118
eGFR estimated GFR, 96
ELISA enzyme-linked immunosorbent assay, 66
EMA European Medicines Agency, 23
EPCR endothelial protein C receptor, 32
ESRD end-stage renal disease, 26
EuroSCORE European system for cardiac operative risk evaluation, 126

F

FDA U.S. Food and Drug Administration, 23
Fe^{2+} ferrous iron, 42
Fe^{3+} ferric iron, 42
FLVCR feline leukaemia virus subgroup C receptor, 43
Fp-1 ferroportin-1, 42

G

G1 first gap phase, 36
G2 second gap phase, 36
GFR glomerular filtration rate, 9
GPX4 glutathione peroxidase 4, 38

H

h hour, 16
HCP1 haem carrier protein 1, 43
HI high-income, 24
HO-1 haem oxygenase-1, 34
HPX haemopexin, 43

I

ICU intensive care unit, 20
IGFBP7 insulin-like growth factor-binding protein 7, 64
IL-13Rα2 IL-13 receptor α2, 49
IL-1β interleukin-1β, 30
iNOS inducible $NO^{\bullet}$ synthase, 31
IQR interquartile range, 84
IRI ischemia-reperfusion injury, 36
IRIS International Renal Interest Society, 28

K

KDIGO Kidney Disease | Improving Global Outcomes, 16

L

LI low-income, 25
LMI lower-middle-income, 25
LOD limit of detection, 87
LOQ limit of quantification, 87
LOS length of stay, 20
LPS lipopolysaccharide, 38
LTf lactoferrin, 42

M

M mitosis, 36
MAS1 proto-oncogene Mas, 14
min minute, 9
MMP matrix metalloproteinase, 31
mo month, 20

N

NADPH nicotinamide adenine dinucleotide phosphate, 31
NF-κB nuclear factor-κB, 30
NGAL neutrophil gelatinase-associated lipocalin, 40
NGALR NGAL receptor, 42
NLRP3 nucleotide-binding domain and leucine-rich repeat family pyrin domain containing 3, 50
NO▪ nitric oxide, 31

O

$O_2^{\bullet-}$ superoxide anion, 31
OD optical density, 84
OR odds ratio, 25

P

PAMP pathogen-associated molecular pattern, 30
PAR-1 protease-activated receptor-1, 33
PC phosphorylcreatine, 18
PETIA particle-enhanced turbidimetric immunoassay, 67
PMDA Japanese Pharmaceuticals and Medical Devices Agency, 23
POCT point-of-care testing, 163
PRR pattern recognition receptor, 30
PSTC Predictive Safety Testing Consortium, 23

R

RAS renin-angiotensin system, 12
RB retinoblastoma, 36
RCD regulated cell death, 34
RFR-G renal functional reserve of the glomerular function, 132
RIPK3 receptor-interacting protein kinase 3, 38
RISK Risk, Injury, Failure, Loss, and End-stage renal disease, 15
ROS reactive oxygen species, 33
RPF renal plasma flow, 9
RRT renal replacement therapy, 24

S

S DNA synthesis phase, 36
SCHI3L1 serum CHI3L1, 75
SCr serum Cr, 15
SD standard deviation, 99
SIRS systemic inflammatory response syndrome, 159
SOFA Sepsis-related Organ Failure Assessment, 96
STROBE strengthening the reporting of observational studies in epidemiology, 65

T

Tf transferrin, 42
TfR1 transferrin receptor protein 1, 42
TG tubuloglomerular, 9
TIMP-2 tissue inhibitor of metalloproteinases-2, 64
TNF-α tumour necrosis factor-α, 30

U

UCHI3L1 urinary CHI3L1, 57
UCr urinary Cr, 67
UMI upper-middle-income, 24
UNGAL urinary NGAL, 63
UO urine output, 15
UTI urinary tract infection, 81

W

w week, 20

Y

y year, 15

Chapter 1

General Introduction

I. The kidneys

I.A. Renal morphology and function

In humans the kidneys are crucial for maintaining the extracellular environment in equilibrium. They excrete some of the waste products of metabolism (e.g., creatinine **(Cr)**), specifically adjust the urinary excretion of water and electrolytes to match net intake and endogenous production, and participate in the regulation of acid-base balance. The basic unit of the kidney is the nephron **(Figure 1)**, with each kidney in humans containing ±1.0-1.3 million nephrons. The formation of an ultrafiltrate of plasma across the glomerulus **(Figure 2)**, termed glomerular filtration, is the initial step in the excretory function of the nephron. The kidney secretes hormones as well. In this way it participates in the regulation of systemic and renal haemodynamics, red blood cell production and bone metabolism. Finally, it performs such miscellaneous functions as gluconeogenesis when fasting and catabolism of peptide hormones [1].

I.B. Regulation of glomerular filtration rate and renal plasma flow

The blood flow to the kidneys (0.95-1.25 l/minute **(min)**) averages 20 % of the cardiac output. In terms of flow per 100 g weight, this is 8 times greater than coronary blood flow. The rate of glomerular filtration **(GFR)** averages 95-125 ml/min (135-180 l/day **(d)**) in a normal adult [2].

Over a wide range of renal arterial pressures the GFR and renal plasma flow **(RPF)** remain roughly constant. This phenomenon has been termed autoregulation. Initially, it is primarily mediated by the stretch receptors in the afferent arterioles (myogenic response) and tubuloglomerular **(TG)** feedback **(Table 1)**. When the renal arterial pressure is substantially reduced, angiotensin II **(Ang II)** dependence is most prominent **(Figure 3)**.

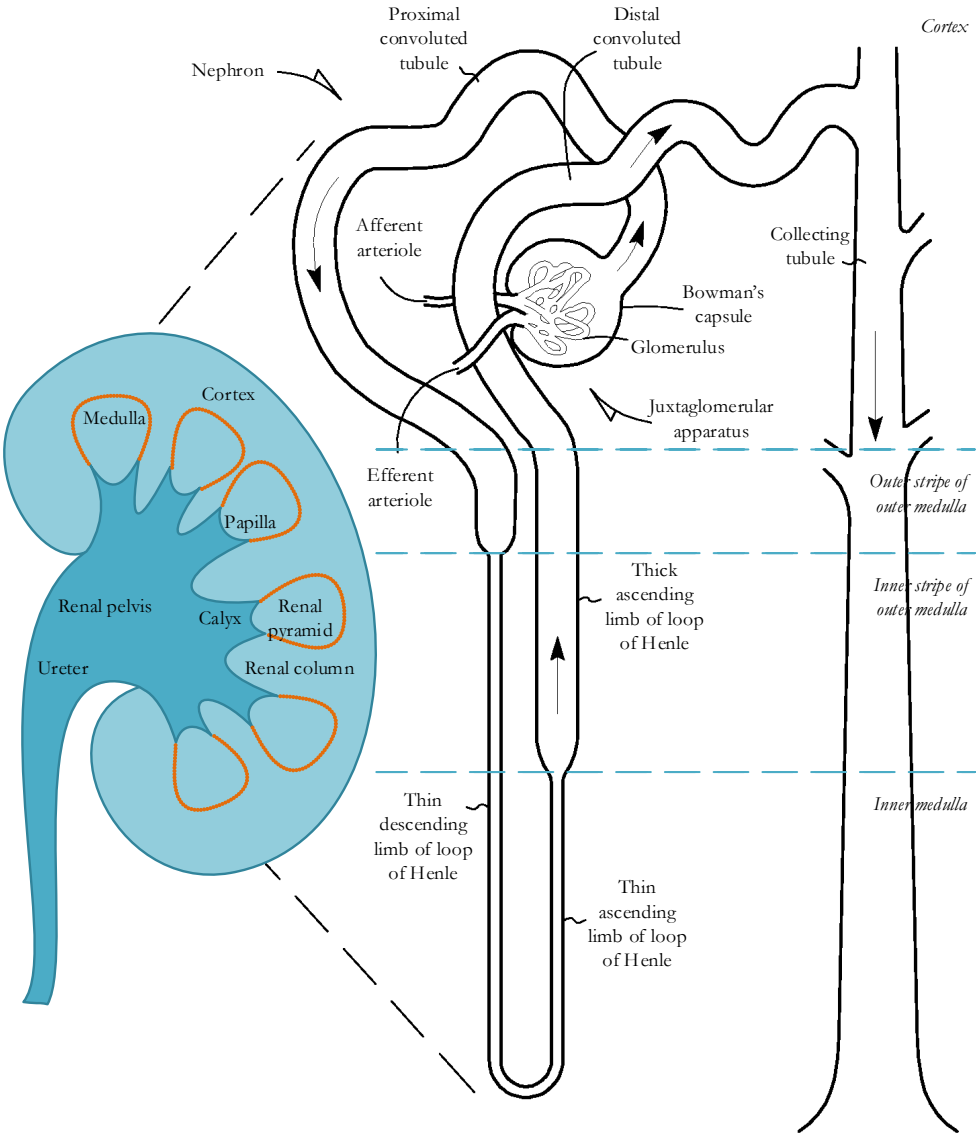


Figure 1 | Section of a human kidney with anatomic relationships of the component parts of the nephron (adapted from [1]; created with Motifolio)

The thick ascending limb has a cortical segment that returns to the region of the parent glomerulus. In this area the specialized tubular cells of the macula densa are located. Together with the renin-secreting juxtaglomerular cells of the afferent arteriole, the macula densa composes the juxtaglomerular apparatus.

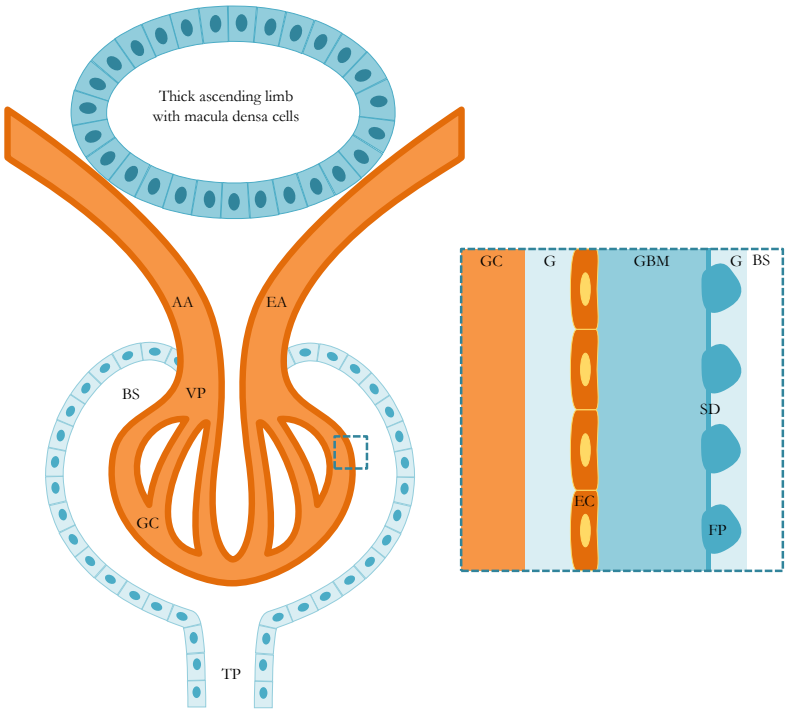


Figure 2 | Anatomy of the glomerulus (based on [3, 4]; created with Motifolio)
Abbreviations: AA afferent arteriole, BS Bowman's space, EA efferent arteriole, EC endothelial cell, FP foot process of podocyte, G glycocalyx, GBM glomerular basement membrane, GC glomerular capillary, SD slit diaphragm, TP tubular pole, VP vascular pole

Autoregulation can be overridden by neurohumoral vasoconstriction in hypovolemic states, in an attempt to maximize cerebral and coronary perfusion. An important role in modifying the vasoconstrictive Ang II and norepinephrine effects is played by renal vasodilator prostaglandins. They attenuate the degree of arteriolar constriction and prevent excessive renal ischemia [2].

TG feedback refers to the alterations in GFR induced by changes in tubular flow rate (especially when renal arterial pressure is increased). In the cortical segment of the thick ascending limb, which returns to the region of the parent glomerulus, the specialized tubular cells of the macula densa are located **(Figures 1 and 2)**. These cells sense changes in the delivery and subsequent reabsorption of chloride **(Table 1)**.

Table 1 | Myogenic response and tubuloglomerular feedback (based on [2])

Stimulus	↑ Renal arterial pressure			
Response	**Myogenic response**		**Tubuloglomerular feedback**	
	↑ Degree of stretch in stretch receptors in the wall of the afferent arteriole ↑ Cell entry of calcium in vascular smooth muscle		↑ GFR ↑ Macula densa chloride delivery and subsequent reabsorption ↑ Cell entry of calcium in vascular smooth muscle	
Effect	**Constriction of the afferent arteriole**		**Constriction of the afferent arteriole**	
	↓ GFR	↑ Renal vascular resistance ↓ RPF	↓ GFR	↑ Renal vascular resistance ↓ RPF

Conversely, as renal arterial pressure decreases, all directions of change are reversed and the afferent arteriole is dilated.
Abbreviations: GFR glomerular filtration rate, RPF renal plasma flow

Besides the stretch receptors in the afferent arteriole and the sympathetic nerves ending in the renin-secreting juxtaglomerular cells, the composition of tubular fluid reaching the macula densa contributes to the regulation of renin[1] secretion as well. The 'renal' or 'systemic' renin-angiotensin system **(RAS)** importantly affects the GFR (**Figure 3)** [1, 2].

Although both afferent and efferent arterioles are constricted by Ang II, the efferent arteriole has a smaller basal diameter, resulting in a greater increase in efferent resistance. The net result on the regulation of GFR is variable in different conditions, as Ang II also sensitizes the afferent arteriole to the constricting signal of TG feedback and constricts the glomerular mesangium (i.e., specialized smooth muscle cells) as well [2].

[1] uniprot/P00797

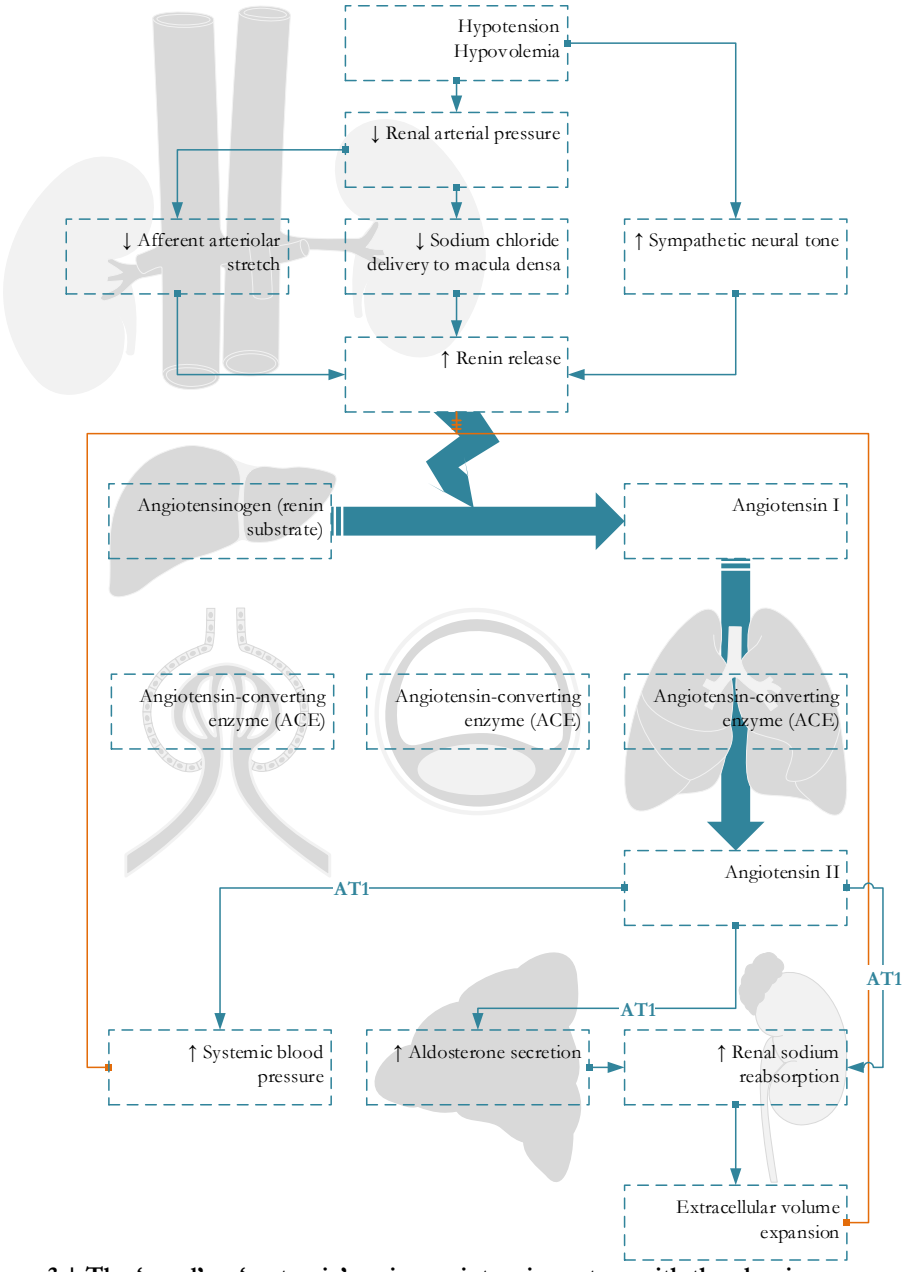


Figure 3 | The 'renal' or 'systemic' renin-angiotensin system with the classic angiotensin-converting enzyme/angiotensin II/type-1 angiotensin II receptor axis (based on [2]; created with Motifolio)
Abbreviations: AT1 type-1 angiotensin II receptor

The effects of Ang II are mediated by the type-1 Ang II[2] **(AT1)** receptor, which is present in practically all tissues. Besides vasoconstriction, many inflammatory and fibrotic effects of Ang II are also a consequence of AT1 receptor activation. However, Ang II can also activate the type-2 Ang II[3] **(AT2)** receptor, which is upregulated in many cases of injury (e.g., to the kidney, heart and brain). The AT2 receptor is part of the protective arm of the RAS, i.e. when activated it has anti-inflammatory and anti-fibrotic effects **(Figure 4)**. In addition, the non-classic angiotensin-converting enzyme 2[4] **(ACE2)**/Ang 1-7/proto-oncogene Mas[5] **(MAS1)** axis has been reported to function as a counterbalance to the classic angiotensin-converting enzyme[6] **(ACE)**/Ang II/AT1 axis **(Figure 4)** [5].

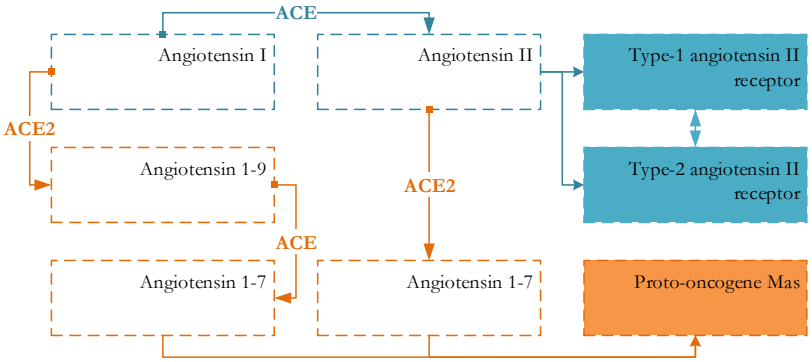


Figure 4 | The non-classic angiotensin-converting enzyme 2/angiotensin 1-7/proto-oncogene Mas receptor axis (based on [5]; created with Motifolio)
The type-2 angiotensin II receptor and the non-classic ACE2/Angiotensin 1-7/proto-oncogene Mas axis are part of the protective arm of the RAS.
Abbreviations: ACE angiotensin-converting enzyme, ACE2 angiotensin-converting enzyme 2, RAS renin-angiotensin system

[2] uniprot/P30556

[3] uniprot/P50052

[4] uniprot/Q9BYF1

[5] uniprot/P04201

[6] uniprot/P12821

II. Acute kidney injury

II.A. Current definitions of acute kidney injury

Acute kidney injury **(AKI)** is an abrupt loss of kidney function that includes, but is not limited to, acute renal failure **(ARF)**. It is a broad clinical syndrome encompassing various aetiologies **(Table 2)** and was described for the first time more than 200 years **(y)** ago [6]. However, AKI did not have a consensus definition until a decade ago with the birth of the 'Risk, Injury, Failure, Loss, and End-stage renal disease' **(RISK)** system [7].

Table 2 | Potential aetiologies of acute kidney injury [8]

Rhabdomyolysis-induced pigment nephropathy
Acute tubular necrosis
▪ Ischemic
▪ Direct injury
Acute interstitial nephritis
Glomerular injury
Thrombotic micro-angiopathy
Malignant hypertension
Vascular injury
▪ Vasculitis
▪ Renal infarct
Obstructive nephropathy

Acute kidney injury represent a very heterogeneous syndrome, with often a multifactorial aetiology including several injurious hits in time.

Before 2004 AKI was still termed ARF. A logical approach to renal 'failure' is to start by defining what it is that the kidney does. However, many of its functions either require complex neurohumoral interactions involving also other organs (e.g., RAS) or are shared with other organs (e.g., acid-base balance with the lung). In fact, there are only two functions that are unique to the kidney, i.e. the production of urine and the excretion of nitrogenous (Cr and urea) waste products [9]. The lack of a precise definition of ARF resulted in more than 30 definitions in the medical literature [7], which caused wide variation in the reported incidence and clinical outcomes of ARF. Most definitions had common elements, including the use of serum Cr **(SCr)** and, often, urine output **(UO)**. In 2004, the RIFLE system opened a new era for the definition of AKI [7]. Revised definitions for the diagnosis and staging of AKI followed, with in 2007 the Acute Kidney

Injury Network **(AKIN)** system [10], and in 2012 the Kidney Disease | Improving Global Outcomes **(KDIGO)** system [11]. KDIGO defines AKI in adults as either increase in SCr by ≥ 0.3 mg/dl within 48 hours **(h)** or increase in SCr to ≥ 1.5 times baseline, which is known or presumed to have occurred within the prior 7 d, or UO of < 0.5 ml/kg/h for 6 consecutive h. Then, AKI is staged for severity according to defined criteria **(Figure 5)**. Evaluation of these systems in many studies has enabled a better understanding of the epidemiology of AKI and has shown a clear association between the severity of AKI and poor outcomes.

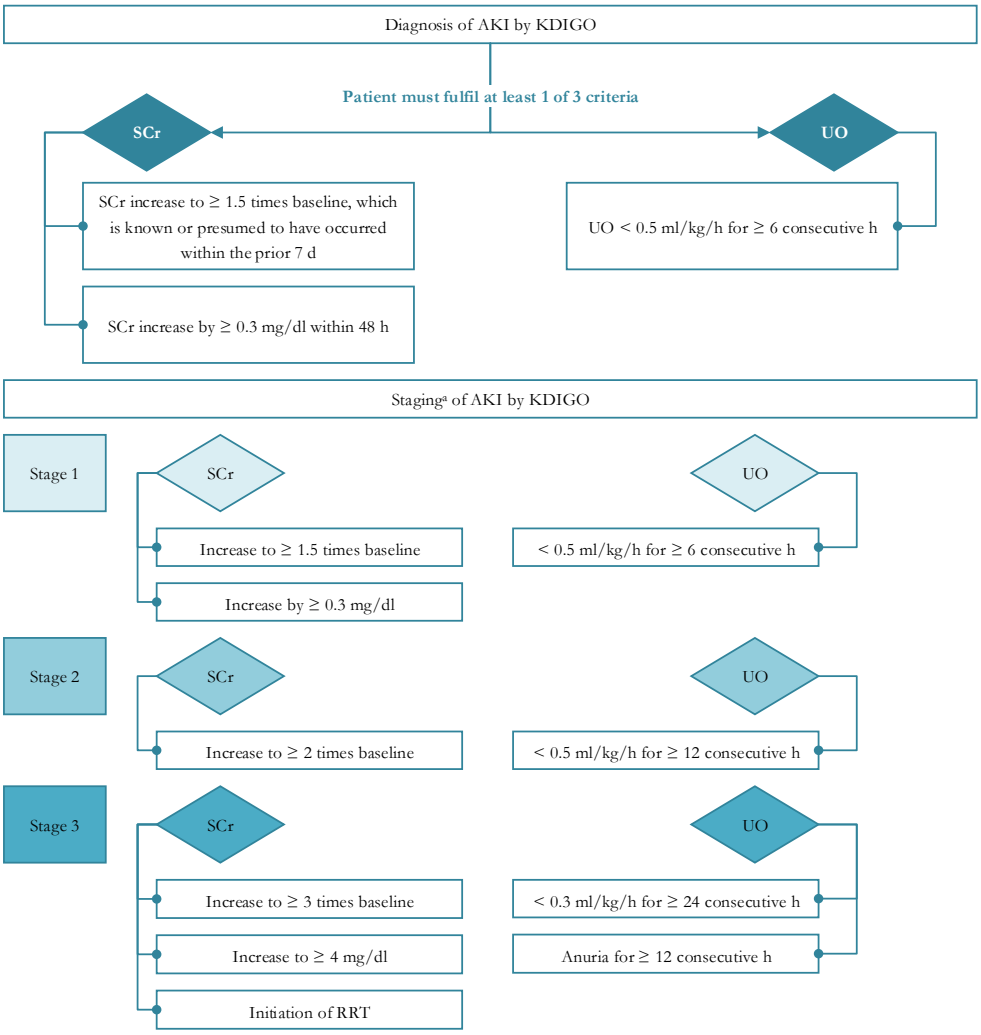


Figure 5 | Kidney Disease | Improving Global Outcomes definition and staging of acute kidney injury in adults (adapted from [11])

[a]For staging purposes, patients should be staged according to the criterion or criteria that give(s) them the highest stage

Abbreviations: AKI acute kidney injury, d day, h hour, KDIGO Kidney Disease | Improving Global Outcomes, RRT renal replacement therapy, SCr serum creatinine, UO urine output

II.A.1. Imperfections in current definitions of acute kidney injury

Current definitions of AKI define acute changes in kidney function using SCr and UO as criteria. These two proxies of the GFR are not perfect in terms of sensitivity and specificity, and clinicians should be alert for making a faulty diagnosis.

II.A.1.i. Background: creatine metabolism and the creatine kinase energy shuttle

The creatine kinase **(CK)** / phosphorylcreatine **(PC)** / creatine **(C)** system participates in energy metabolism. In tissues devoid of CK and PC, like liver, it is assumed that high-energy phosphate transport between sites of adenosine triphosphate **(ATP)** production (mitochondria) and ATP consumption (cytosol) relies on diffusion of ATP and adenosine diphosphate **(ADP)** alone. This is, however, clearly inadequate for CK-containing tissues, like skeletal or cardiac muscle, brain, retina and spermatozoa, with high and fluctuating energy demands. The daily demand for C is met either by intestinal absorption of dietary C or by de novo C biosynthesis **(Figure 6)**. In mammals the main route of C biosynthesis involves formation of guanidinoacetate in the kidney, its transport through the blood, and its methylation to C in the liver. Up to 94% of C is found in muscular tissues **(Box 1)**, where C has to be taken up from the blood against a large concentration gradient by the saturable sodium- and chloride-dependent C transporter 1[7] **(Figure 6)**. The muscular C and PC are non-enzymatically and irreversibly converted at an almost steady rate (±1.7 % of total C and PC per day) to Cr, which diffuses out of the cells and is excreted by the kidneys into the urine **(Figure 6)** [12].

[7] uniprot/P48029

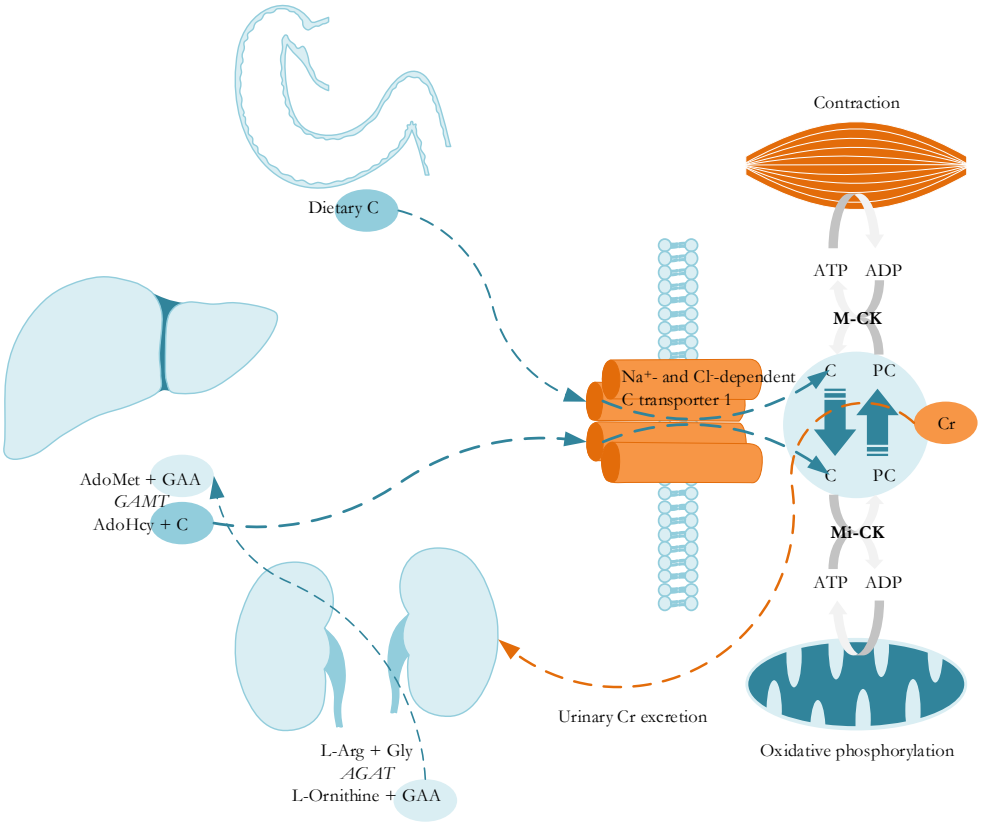
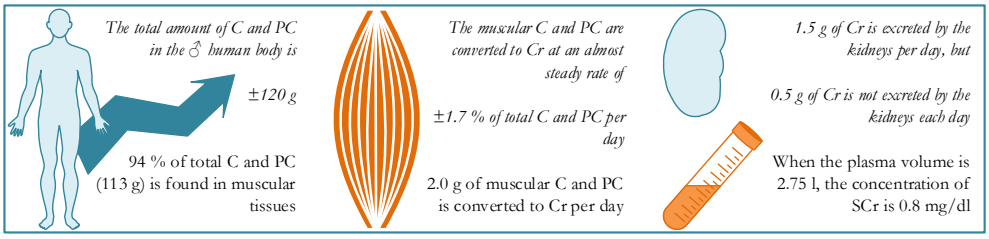


Figure 6 | Major routes of creatine metabolism and the creatine kinase energy shuttle in the mammalian body (based on [12, 13]; created with Motifolio)
Abbreviations: AdoHcy S-adenosyl-L-homocysteine, AdoMet S-adenosyl-L-methionine, ADP adenosine diphosphate, AGAT L-arginine:glycine amidinotransferase, ATP adenosine triphosphate, C creatine, CK creatine kinase, Cl^- chloride, Cr creatinine, GAA guanidinoacetic acid (guanidinoacetate), GAMT S-adenosyl-L-methionine:N-guanidinoacetate methyltransferase, Gly glycine, L-Arg L-arginine, M-CK cytosolic muscle-type CK isoenzyme, Mi-CK mitochondrial CK isoenzyme, Na^+ sodium, PC phosphorylcreatine

Box 1 | The creatine metabolism in the human body (based on [12]; created with Motifolio)



Abbreviations: C creatine, Cr creatinine, PC phosphorylcreatine

II.A.1.ii. Imperfections of serum creatinine and urine output

The validity of SCr as proxy of the GFR critically depends on the assumptions that Cr is produced at a steady rate, is physiologically inert and is excreted solely by glomerular filtration in the kidney [12]. However, these assumptions are not by default valid in each patient.

Age, gender, ethnicity and dietary protein intake all affect muscle mass and, therefore, Cr production. After the age of 30 y, adults lose 3-8 % of their muscle mass per decade [14]. Further, lean tissue loss is largely inevitable during prolonged bed rest. Inactivity-induced loss of muscle mass is most rapid during the initial d or weeks **(w)** of inactivity and predominantly affects the lower body musculature [14]. A loss of 0.4 kg of lean leg mass was reported in healthy young adults following 28 d of bed rest [15]. In healthy older adults a loss of 0.95 kg of lean leg mass was reported following just 10 d of bed rest [16]. Importantly, an increased stress response (e.g., hypercortisolemia) may also contribute to muscle loss during periods of impaired physical activity. Compared with eucortisolemic controls, pharmacologically-induced hypercortisolemia in healthy young adults resulted in a 3-fold greater loss of lean leg mass following 28 d of bed rest [15]. Consequently, a false-low SCr concentration due to muscle wasting and fluid overload is common in critically ill patients with a long intensive care unit **(ICU)** length of stay **(LOS)**. In addition, many patients will present without a reliable baseline SCr on record. KDIGO suggests that in patients without haemodilution secondary to massive fluid resuscitation, the lowest SCr obtained during a hospitalization should be used to diagnose and stage AKI [11]. Assuming that the illness and LOS were not too long, this SCr would then usually be equal to or greater than the baseline [11]. Alternatively, the international and interdisciplinary organization Acute Dialysis Quality Initiative **(ADQI)** recommends that a reliable baseline SCr concentration may be obtained within a 3-month **(mo)** period preceding the current event in patients who are in stable condition [17, 18].

Although extrarenal Cr clearance may be negligible in healthy individuals, which led to the postulate that Cr is physiologically inert, it may become highly relevant in chronic kidney disease **(CKD)** patients. Most likely, Cr is excreted into the gut where it is converted by bacterial creatininase to C. In turn, C is retaken up into the blood [12]. This enteric cycling may limit Cr toxicity, as there are two oxidative Cr degradation pathways as well. One leads to the formation of methylguanidine and the other to methylurea, which are both uremic toxins [12]. In CKD patients, an increasing proportion of Cr is excreted by tubular secretion rather than glomerular filtration as well [12]. These factors may result in overestimation of the GFR.

Importantly, accurate estimation of GFR from the serum level of an endogenous filtration marker, like Cr, requires a steady state. After an acute GFR decline, generation of Cr is unchanged, but filtration is reduced, resulting in retention of Cr (a rising positive balance) and a rising plasma level (non-steady state). Although GFR remains reduced, the rise in plasma level leads to an increase in filtered load until filtration equals generation (new steady state). In the non-steady state, the direction of change in SCr indicates the direction of change in GFR, and the rate of change in SCr provides some indication of the magnitude of change in GFR [19].

Meanwhile, UO is an imperfect proxy of the GFR as well, obviously because it is affected by diuretic use [20, 21]. In addition, when patients are fluid-loaded, a varying amount of plasma water may be removed by glomerular filtration, increasing UO [22, 23]. Another issue is that even in catheterized patients, urine volume measurements may be inaccurate. For example, a decreased urine volume may be caused by physical obstruction either internally (e.g., urinary catheter debris) or externally (e.g., urinary catheter kinking) to the urinary catheter. Also, for the nurses it may be difficult to provide exactly timed 1-h registrations of urine volume, complicating accurate measurement of UO [22, 23].

II.A.2. Rationale for the urgent need for novel biomarkers that are not proxies of the GFR

Because there is no single proven intervention for AKI, clinicians may be hesitant to change the 'status quo' concerning the diagnosis of AKI. Nevertheless, single center studies have shown that more early recognition of AKI combined with some simple preventive or supportive measures may lower the progression of less severe to severe AKI. The first study providing an intervention-based clinical rationale for using more sensitive definitions of AKI was the 'early renal service involvement (EARLI)' pilot study [24], showing that a 1-time nephrology consultation within 18 h of the onset of mild AKI decreased the incidence of subsequent more severe AKI from 12.9 % in the control group to 3.3 % in the intervention group (P = 0.020). Later on, the first study evaluating the impact of an electronic real-time AKI sniffer on the timeliness and number of preventive or supportive measures for AKI, and on the development of AKI in the ICU [25], showed that in the alert group more patients with RIFLE class 'Risk' had an improved RIFLE class within 8 h (65.9 %) than in the pre- (61.0 %) and post-alert (63.1 %) control groups. However, this benefit could not be demonstrated in patients with RIFLE class 'Injury' or 'Failure', and no beneficial effect on outcome parameters could be found. In 2017 Meersch et al. showed that the use of an AKI care bundle (i.e., intervention therapy; **Table 3**) vs. a standard care bundle (i.e., control therapy; **Table 3**) targeted at a cohort of adult cardiac surgery patients at high risk for AKI – as identified by a novel biomarker that is not a proxy of the GFR – could reduce the occurrence and severity of AKI [26]. A pilot study that used the same biomarker for risk stratification in adult ICU patients after major elective non-cardiac surgery could demonstrate a reduced occurrence and severity of AKI in the intervention group (AKI care bundle) compared to the control group (standard care bundle) in a subgroup (based on biomarker concentration) analysis [27]. Despite the limitations of these studies – e.g., single-center setting, limited number of patients studied – they give us a strong suggestion that there is an association between early management of AKI and beneficial patient and kidney outcomes,

and so provide convincing rationale for using more sensitive definitions of AKI. Moreover, it is likely that the current diagnostic criteria are still not sensitive enough. In this context, following drug toxicity studies and analysis of biomarker performance by the Predictive Safety Testing Consortium's **(PSTC)** Nephrotoxicity Working Group, the first formal qualification of renal safety biomarkers that are not proxies of the GFR for regulatory decision making (2008-2010) marks a milestone in the application of biomarkers to drug development [28]. PSTC brings together pharmaceutical companies and academic institutions to share and validate innovative safety testing methods. The consortium's mission is to identify new and improved safety testing methods and submit them for formal regulatory qualification by the U.S. Food and Drug Administration **(FDA)**, the European Medicines Agency **(EMA)** and the Japanese Pharmaceuticals and Medical Devices Agency **(PMDA)**.

Table 3 | The 'Kidney Disease | Improving Global Outcomes care bundle' to reduce the risk of acute kidney injury among adult patients who have undergone cardiac surgery (based on [26, 29])

Surgery	48 h	72 h
Avoidance of nephrotoxic agents		
Withholding of ACE inhibitors and ARBs		
Close monitoring of SCr and UO		
Avoidance of hyperglycaemia		
Consider alternatives to radio-contrast agents		
Optimization of volume status and haemodynamic parameters		

Volume status and haemodynamics were optimized using stroke volume variation, cardiac output and MAP. Patients in the control group received standard of care: MAP was kept > 65 mmHg and CVP between 8 and 10 mmHg.
Abbreviations: ACE angiotensin-converting enzyme, ARB type-1 angiotensin II receptor blocker, CVP central venous pressure, MAP mean arterial pressure, SCr serum creatinine, UO urine output

II.B. The incidence of acute kidney injury is high and still increasing

The incidence of AKI has increased over the years [30, 31]. Do these changes reflect actual increases in disease incidence, or rather changes in consensus definition, in alerting (e.g., real-time electronic alerting), in coding, or in use of renal replacement therapy **(RRT)**? In their retrospective multicentre study conducted in Canada, Wald et al. observed a near-quadrupling in the incidence of RRT-requiring AKI among critically ill adults over 15 y **(Figure 7)** [32]. This study nicely illustrates that increases in AKI are indeed occurring, which can be attributed to population aging, severe comorbidities, and increased use of modifiable risk factors (e.g., nephrotoxic agents) [31].

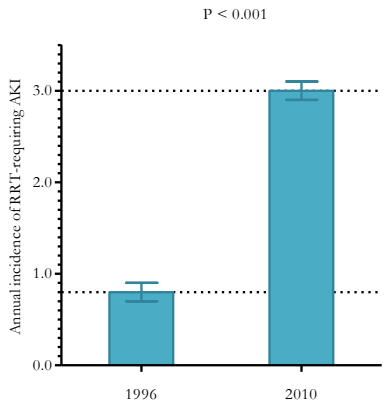


Figure 7 | Increasing incidence of RRT-requiring AKI among critically ill adults from 1996 to 2010 (based on [32]; created with GraphPad Prism)
P for trend is shown. Incidence is expressed as a percentage.

Most available data for AKI epidemiology come from high-income **(HI)** and upper-middle-income **(UMI)** countries. In a recent meta-analysis of studies primarily conducted in hospital settings, among the 147 studies (n = 3,212,925) that adopted a KDIGO-equivalent SCr-based AKI definition, the pooled incidence rate of AKI was 21.0 % in adults (95 % confidence interval **(CI)**: 18.7-23.6 %) [33, 34]. 97.3 % of the studies were from HI (84.4 %) and UMI countries.

The AKI incidence in the ICU is extremely high, i.e. up to 67.2 % in adults [35]. Consistent with this, the multinational cross-sectional AKI-EPI study (n = 1,802) found a similar AKI incidence of 57.3 % for adult ICU patients [36]. 74.9 % of the patients were from HI countries, 16.2 %

from UMI and 8.9 % from lower-middle-income **(LMI)** countries. No significant difference in the occurrence of AKI was found between patients from HI, UMI and LMI countries (P = 0.264).

As 85 % of the world's population resides in low-income **(LI)** and LMI countries, the multinational cross-sectional International Society of Nephrology 0by25 Global Snapshot study enrolled 4,018 adult (91.2 %) and paediatric AKI patients (31.4 % from HI, 39.9 % from UMI, 23.8 % from LMI and 4.9 % from LI countries) from both hospital and non-hospital settings [37]. 58.2 % of these AKI patients had community-acquired AKI. A significant difference in the occurrence of community-acquired AKI was found between LI+LMI countries (77.1 %) and both HI (50.2 %) and UMI (50.8 %) countries. Further, AKI was significantly more severe at diagnosis in patients from LI+LMI countries (57.8 % stage 3) compared with patients from both HI (46.7 % stage 3) and UMI (41.0 % stage 3) countries. In the younger AKI patients from LI+LMI countries, animal envenomation, infections, dehydration and complicated pregnancy were the most important drivers of AKI. By contrast, cardiac failure, surgery and nephrotoxic agents predominantly drove AKI in the older comorbid patients from both HI and UMI countries.

II.C. Patients show poor outcomes after acute kidney injury

II.C.1. High mortality

In the above-mentioned meta-analysis by Susantitaphong et al. [33, 34], among the 106 studies that used a KDIGO-equivalent SCr-based AKI definition and assessed mortality, the pooled mortality rate was 23.3 % for adults with AKI (95 % CI: 21.3-25.5 %). In the AKI-EPI study increasing AKI severity in ICU patients was associated with hospital mortality (median hospital LOS = 14 d) when adjusted for other variables: odds ratio **(OR)** of stage 1 = 1.679 (95 % CI: 0.890-3.169), OR of stage 2 = 2.945 (95 % CI: 1.382-6.276), and OR of stage 3 = 6.884 (95 % CI: 3.876-12.228) [36]. No significant difference in the hospital mortality rate was found between

AKI patients from HI, UMI and LMI countries (P = 0.457; hospital mortality rate within AKI patients of all countries = 26.9 %) [36]. The 0by25 Global Snapshot study reported that in patients with community-acquired AKI, the mortality rate at 7 d was significantly higher in LI+LMI countries compared with both HI and UMI countries [37]. The mortality rate at 7 d within all AKI patients was 11.0 % [37].

Retrospective data from 32,045 adult ICU patients, of which 74.5 % developed AKI, showed that risk for both short- (hospital) and long-term (1 y) outcomes (age-adjusted survival and freedom from RRT) was highest for patients that had any stage of AKI defined by both SCr and UO criteria [38]. Duration of AKI was also a significant predictor of long-term outcomes irrespective of severity [38]. Importantly, Rimes-Stigare et al. demonstrated that even severe de novo AKI (i.e., AKI that is not superimposed on CKD) was independently associated with increased long-term risk for death [39]. Further, Uchino et al. showed that even transient AKI, defined as SCr reduced to 'no AKI' range within 72 h of the onset, was an independent predictor of hospital mortality (OR = 2.264; 95 % CI: 1.856-2.762) [40]. Yet, the majority of these patients with transient azotaemia were classified as RIFLE stage Risk (74.1 %).

II.C.2. Association with chronic kidney disease and cardiovascular disease

Patients who survive an episode of AKI are at risk for progression to CKD, as well as for major adverse cardiovascular events [41, 42]. Importantly, Rimes-Stigare et al. demonstrated that even severe de novo AKI was independently associated with increased long-term risk for not only death, but also CKD and end-stage renal disease **(ESRD)**[39]. Further, Horne et al. demonstrated that even less severe AKI can have significant long-term effects on renal function and proteinuria [43]. In the recent review by Vanholder et al., the authors state that "the treatment of CKD and of ESRD imposes substantial societal costs" [44]. They emphasise that "costs for CKD are not limited to RRT, but also include non-renal healthcare costs, costs not related to healthcare, and costs for patients with CKD who are not yet receiving RRT." In 2001

Van Biesen et al. calculated the annual expenditure per ESRD patient in Belgium by modality of RRT **(Table 4)** [45]. Renal transplantation imposes the lowest societal cost while offering the highest quality of life [44]. Conversely, in-hospital haemodialysis imposes the highest cost and provides the lowest quality of life [44]. Importantly, in the UK the yearly expense (during 2009 and 2010) to the National Health Service for patients with CKD not requiring RRT almost matched that for ESRD patients treated with RRT [46].

Table 4 | Annual expenditure per end-stage renal disease patient in Belgium by modality of renal replacement therapy (2001) (based on [45])

Modality of RRT	Cost in euro	
Haemodialysis	71,790	
Peritoneal dialysis	45,000	
Renal transplantation	Surgery 24,356	First post-operative year 12,810

Abbreviations: RRT renal replacement therapy

Recovery from AKI has recently been investigated by ADQI [47], as there was still a lack of a consensus definition. ADQI states that "persistent AKI is characterized by the continuance of AKI by SCr or UO criteria (as defined by KDIGO) beyond 48 h from AKI onset. Complete reversal of AKI by KDIGO criteria within 48 h of AKI onset characterizes rapid reversal of AKI. Although the optimal duration of sustained AKI reversal is unknown, a minimum of 48 h is necessary to separate two distinct AKI episodes. Persistent AKI frequently becomes acute kidney disease **(AKD)**, defined as a condition wherein KDIGO criteria for AKI stage ≥ 1 persist ≥ 7 d after an exposure. AKD that persists beyond 90 d is considered to be CKD."

II.D. Acute kidney injury in veterinary medicine of companion animals

In humans, the KDIGO system was developed to stratify the extent and duration of AKI. Unfortunately, KDIGO-like criteria are not as consistently applicable in dogs and cats because AKI in pets most commonly develops outside the hospital setting. Consequently, the abruptness of the disease and the magnitude of changes in SCr and UO are rarely known or quantitated. A canine and feline AKI grading **(Table 5A)** and subgrading scheme **(Table 5B)** was developed by

Cowgill and accepted by the International Renal Interest Society **(IRIS)**, provisionally in 2012 and finally in 2013 (revised in 2016) [48]. IRIS was created in 1998 to advance the scientific understanding of kidney diseases in dogs and cats. It defines five AKI grades based on SCr. Each grade is further subdivided on the basis of UO (either oligo-/anuric or non-oliguric) and requirement for RRT. This scheme provides an instrument for the more early recognition and assessment of outcomes of AKI in dogs and cats.

Table 5A | International Renal Interest Society grading criteria for acute kidney injury in dogs and cats (based on [48])

AKI grade	SCr (mg/dl)	Clinical description			
I	≤ 1.6	Non-azotaemic AKI documented by			
		Historical evidence	Clinical evidence	Laboratory evidence	Imaging evidence
			Including measured oliguria (< 1 ml/kg/h) or anuria over 6 h	Including progressive non-azotaemic increase in SCr of ≥ 0.3 mg/dl within 48 h	
			Including volume responsiveness[a]	Including volume responsiveness[b]	
II	> 1.6	Mild AKI documented by			
		Historical evidence	Clinical evidence	Laboratory evidence	Imaging evidence
			Including measured oliguria (< 1 ml/kg/h) or anuria over 6 h	Including progressive azotaemic increase in SCr of ≥ 0.3 mg/dl within 48 h	
			Including volume responsiveness[a]	Including volume responsiveness[b]	
III	> 2.5	Documented moderate to severe AKI			
IV	> 5.0				
V	> 10.0				

[a]Defined as an increase in UO to ≥ 1 ml/kg/h over 6 h
[b]Defined as a decrease in SCr to baseline over 48 h
Abbreviations: AKI acute kidney injury, SCr serum creatinine, UO urine output

Table 5B | International Renal Interest Society subgrading of acute kidney injury (based on [48])

AKI grade	SCr (mg/dl)	Subgrade			
I	≤ 1.6	Non-oliguric (NO)	Non-oliguric (NO) with RRT need	Oligo-/anuric (O)	Oligo-/anuric (O) with RRT need
II	> 1.6				
III	> 2.5				
IV	> 5.0				
V	> 10.0				

Abbreviations: AKI acute kidney injury, SCr serum creatinine

The most common aetiologies of AKI in dogs and cats are listed in **Table 6**. Unfortunately, a definitive underlying aetiology for development of AKI is often not found.

Table 6 | Common aetiologies of acute kidney injury in dogs and cats (based on [49])

Dogs	Dogs and cats	Cats
Leptospirosis **(2.0 %)**	Pyelonephritis **(2.0 %)**	Ureteral obstruction****
Grape or raisin toxicity	Nephrotoxic drugs (e.g., NSAIDs) **(9.1 %)**	Lily toxicity[a]
Acute pancreatitis*** (9.1 %)**	Ethylene glycol **(12.1 %)**	Renal lymphoma**** (1.0 %)**
Lyme nephritis	Severe blood pressure alterations (low or high) **(24.2 %)**	

[a]Lilies (plants) considered potentially nephrotoxic to cats are Easter lily, Stargazer lily, Japanese Show lily, Tiger lily, Asiatic lily, Leopard lily, Trumpet lily, Panther lily, White lily, Yellow lily, Day lily and Calla lily [50].
***Indicates that the disorder can occur in both dogs and cats, but occurs much more commonly in dogs
****Indicates that the disorder can occur in both dogs and cats, but occurs much more commonly in cats
The percentage of AKI dogs that were identified with the disorder is given (retrospective study in 99 AKI dogs; [51]). Additionally, 18.2 % of the AKI dogs were identified with multiple disorders, while in 22.2 % no disorder was identified.

Harison at al. investigated the incidence of hospital-acquired AKI in dogs and cats (i.e., patients with an initial SCr of > 1.6 mg/dl were excluded). This retrospective study (n = 400 for dogs, n = 128 for cats) reported an AKI incidence of 15 % in dogs and 21 % in cats based on an acute (non-)azotaemic increase in SCr of ≥ 0.3 mg/dl within 48 h [52].

III. Increased understanding of the pathophysiology of acute kidney injury

As logical approach to other biological parameters for AKI than SCr and UO, the following paragraphs summarize the latest understanding of the pathophysiology of AKI. The cellular mechanisms behind AKI are reviewed, including cellular activation, hibernation and suicide.

III.A. Cellular mechanisms

III.A.1. Cellular activation of different cell types

III.A.1.i. Macrophages

A variety of pathogen-associated molecular patterns **(PAMP)** (i.e., proteins, sugars, lipids or nucleic acids unique to pathogens) and damage/danger-associated molecular patterns **(DAMP)** (i.e., molecules like heat-shock proteins and ATP that come mainly from distressed or dead host cells) activate the innate immune response by binding to pattern recognition receptors **(PRR)** on macrophages **(Figure 8)** [53]. Activated macrophages secrete tumour necrosis factor-α **(TNF-α)** and interleukin-1β **(IL-1β)**, which – after binding to their receptors – lead to cellular activation [54].

While IL-1β precursor is produced by, inter alia, macrophages in response to a nuclear factor-*κ*B (**NF-*κ*B)** (transcription factor) activating stimulus (e.g., PAMP/DAMP binding to PRR, TNF-α and IL-1 itself), the IL-1α precursor is constitutively present in, inter alia, endothelial cells and renal tubular cells [55]. Unlike the IL-1β precursor, the IL-1α precursor is fully active and functions as an "alarmin" released upon regulated necrosis [55].

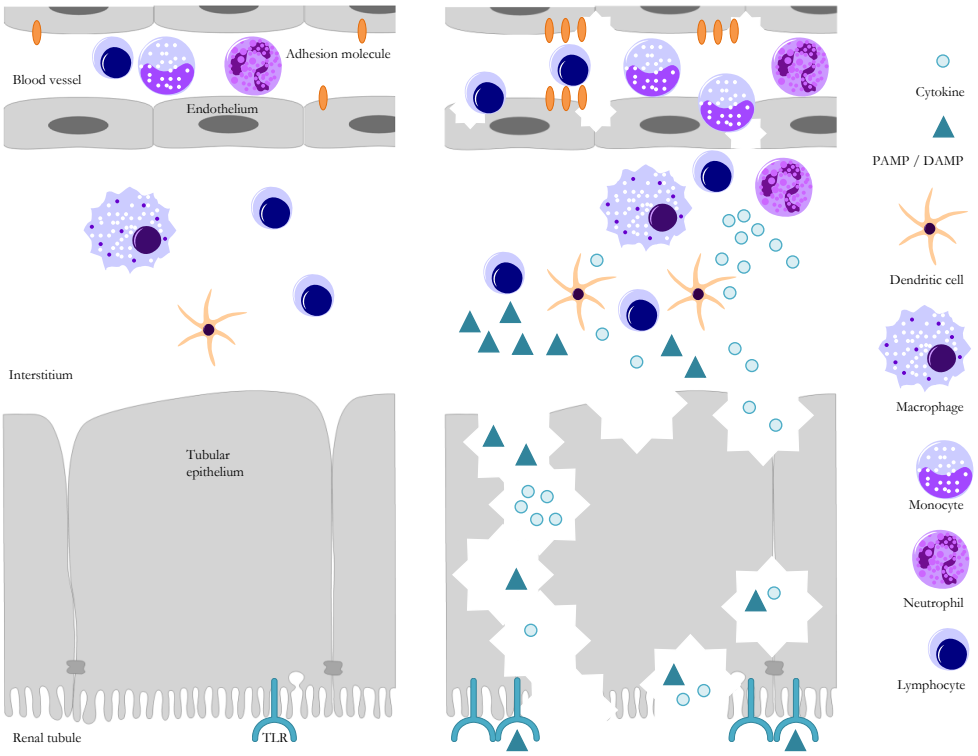


Figure 8 | Major effector cells of both the innate and the adaptive immune system contribute to the establishment of acute kidney injury (based on [56]; created with Motifolio) The *left panel* shows a normal kidney, while the *right panel* shows a kidney with AKI. Dendritic cells act as messengers between the innate and adaptive immune systems. Note that adhesion molecules mediate lymphocyte capturing and rolling.
Abbreviations: AKI acute kidney injury, DAMP damage/danger-associated molecular pattern, PAMP pathogen-associated molecular pattern, TLR Toll-like receptor (a type of pattern recognition receptor)

Although innate responses do have some specificity in recognizing pathogens, their effector molecules kill non-specifically. Activated macrophages produce superoxide anion **($O_2^{\bullet-}$)** from preformed nicotinamide adenine dinucleotide phosphate **(NADPH)** oxidase enzymes, nitric oxide **($NO^{\bullet}$)** gas from arginine by means of inducible $NO^{\bullet}$ synthase **(iNOS)** and various matrix metalloproteinases **(MMP)** [53]. Therefore, whenever innate immunity kills, it also causes collateral damage to normal tissues (e.g., MMPs degrade extracellular matrix components like collagen [53]). Within the kidney, the glomerular basement membrane, the specialized cell-to-cell

junctions of the podocytes, called slit diaphragms, and the endothelial and podocyte glycocalyx are harmed, resulting in vascular leakage and interstitial oedema **(Figure 2)** [57]. This increased permeability allows haemo-concentration, which in turn increases the endothelial-leukocyte interactions [54]. The outcome is amplification of the pro-inflammatory signal, leading to further dysfunction of the endothelial permeability barrier [54].

III.A.1.ii. Kidney endothelial cells

Endothelial cells release a variety of pro-inflammatory mediators (i.e., cytokines and chemokines) upon TNF-α or IL-1 stimulation. The chemokine IL-8 plays a key role in neutrophil chemotaxis and degranulation, whereas C-C motif chemokine 2 **(CCL2)** (alternative name: monocyte chemoattractant protein 1 or MCP-1) is involved in attracting monocytes **(Figure 8)** [54]. Fusion of Weibel-Palade bodies (storage granules of endothelial cells), containing P-selectins[8], with the cell membrane and synthesis of E-selectins[9] are mediated by TNF-α or IL-1 as well [54]. These adhesion molecules mediate lymphocyte capturing and rolling by binding to the lymphocyte adhesion receptor L-selectin[10] **(Figure 8)** [58]. Activated endothelial cells also activate coagulation, which is central to microcirculatory dysfunction [54]. Activation of the endothelial NF-κB pathway reduces thrombomodulin[11] and endothelial protein C receptor[12] **(EPCR)** transcription and increases their shedding from the endothelial surface [59]. Such activation impairs the activated protein C **(aPC)** anti-coagulant and anti-inflammatory mechanisms. Under physiological conditions, EPCR binds circulating protein C[13] **(cPC)**, synthesized in the liver, and presents it to the thrombin[14]-thrombomodulin complex, generating aPC **(Figure 9)**. Besides

[8] uniprot/P16109

[9] uniprot/P16581

[10] uniprot/P14151

[11] uniprot/P07204

[12] uniprot/Q9UNN8

[13] uniprot/P04070

[14] uniprot/P00734

interrupting the coagulation cascade by cleavage of factors Va[15] and VIIIa[16], aPC also activates anti-inflammatory properties by coupling of the aPC-EPCR complex to protease-activated receptor-1[17] **(PAR-1)** [59].

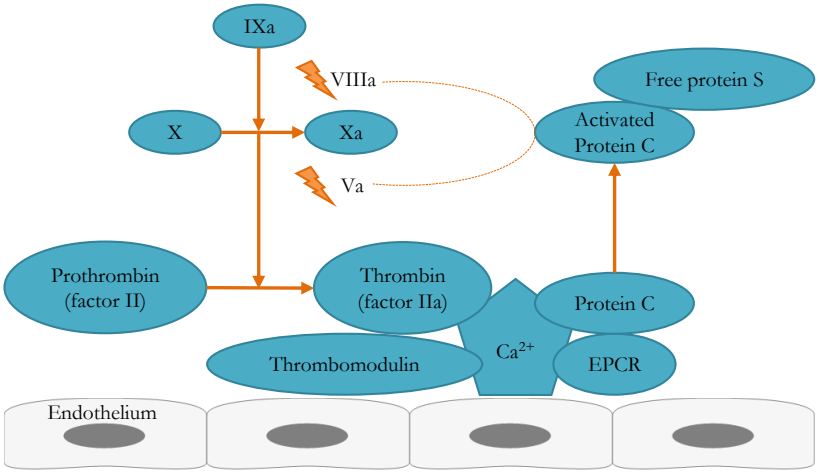


Figure 9 | Anticoagulant mechanisms of the protein C system (based on [60]; created with Motifolio)
Protein S acts as a cofactor of activated protein C and the complex inhibits factors VIIIa and Va. Protein C is activated by the EPCR bound to the complex thrombin (factor IIa)-thrombomodulin. Solid lines denote activation and broken lines inhibition.
Abbreviations: Ca^{2+} calcium, EPCR endothelial protein C receptor

III.A.1.iii. Kidney tubular epithelial cells

An organized interaction between PAMPs/DAMPs and the tubular epithelial cell occurs in the S1 segment of the proximal tubule. The findings of Kalackeche et al. suggest that activation of surveillance receptors, or PRRs, increases reactive oxygen species **(ROS)** through the oxidation of NADPH by NADPH oxidase enzymes [61]. As essential second messengers, ROS are required for the release of pro-inflammatory cytokines (e.g., TNF-α) to effect an appropriate innate immune response [62]. The S1 segment would thus act as the 'sensor' of PAMPs and

[15] uniprot/P12259

[16] uniprot/P00451

[17] uniprot/P25116

DAMPs in the filtrate and, as such, auto-protect itself (i.e., upregulation of cytoprotective molecules with antioxidant properties, like haem oxygenase-1[18] **(HO-1)**), while simultaneously signalling to neighbouring segments (i.e., paracrine fashion through secretion of TNF-α), resulting in an organized oxidative outburst in these epithelial cells of the adjacent proximal tubular segments (S2 and S3) **(Figure 10)** [61]. Further, it is hypothesized that this oxidative outburst is the trigger for S2 and S3 proximal tubular epithelial cellular hibernation *(cf. section **III.A.2.** "Cellular hibernation of kidney tubular epithelial cells")*, which is an adaptive response [61].

III.A.2. Cellular hibernation of kidney tubular epithelial cells

Gomez et al. hypothesize that the kidney tubular epithelial cells of specifically the S2 and S3 segments of the proximal tubule, coordinate a response to the oxidative outburst directed by S1 proximal tubular epithelial cells [63]. This adaptive response, i.e. reprioritization of energy-consumption, autophagy and cell-cycle arrest, would avoid triggering regulated cell death **(RCD)** *(cf. section **III.A.3.** "Cellular suicide of kidney tubular epithelial cells")* [63].

As part of the cellular response to threatening circumstances, a highly conserved mechanism across species is that ATP-consuming processes have a hierarchy of response dependent on the level of energy (i.e., ATP) charge or supply, avoiding energy expenditure in non-essential pathways, and allowing the cell to prioritize energy-consumption in essential processes [64]. During AKI both relocation of the sodium-potassium ATPase pump[19] [65], and downregulation of the megalin receptor[20] reduce cellular energy demands [66], thereby causing an important reduction in oxygen consumption.

[18] uniprot/P09601

[19] uniprot/P05023 and uniprot/P50993 and uniprot/P13637 and uniprot/Q13733

[20] uniprot/P98164

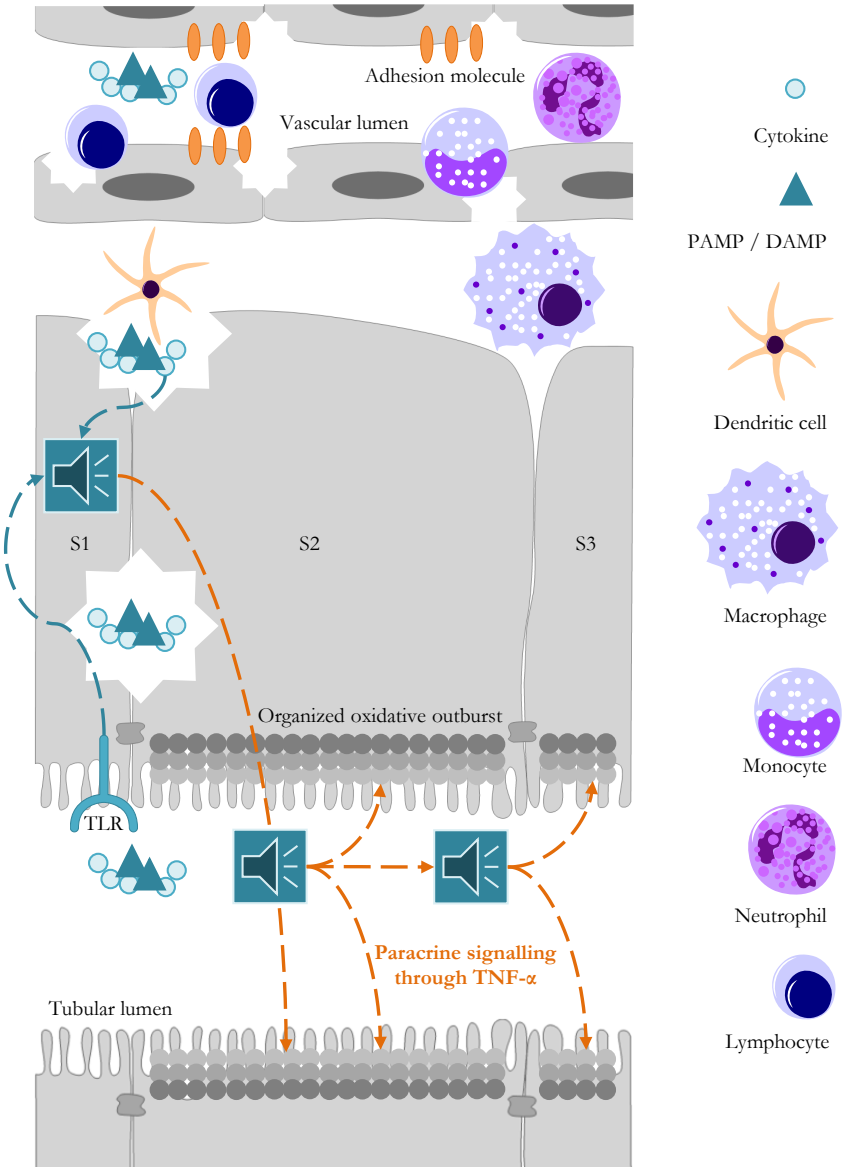


Figure 10 | The S1 segment as the 'sensor' of pathogen-associated molecular patterns and damage/danger-associated molecular patterns in the filtrate (based on [63]; created with Motifolio)

Simultaneously, the S1 segment signals to neighbouring segments (i.e., paracrine fashion through secretion of TNF-α), resulting in an organized oxidative outburst in these epithelial cells of the adjacent proximal tubular segments (S2 and S3).

Abbreviations: DAMP damage/danger-associated molecular pattern, PAMP pathogen-associated molecular pattern, TLR Toll-like receptor (a type of pattern recognition receptor), TNF-α tumour necrosis factor-α

The evolutionarily conserved multi-step process of autophagy was traditionally viewed as a non-selective sequestration of the cytoplasmic contents in autophagosomes and its degradation by lysosomes [67]. Selective autophagy has, however, been reported (e.g., for the elimination of damaged mitochondria, termed mitophagy) [67]. Per milligram of tissue, only the heart exceeds the kidney's abundance of mitochondria [68]. Not surprisingly, mitophagy was increased in kidney tubular epithelial cells *in vivo* in ischemia-reperfusion injury **(IRI)** models of AKI [69, 70].

Four distinct phases compose the eukaryotic cell-cycle: the first gap phase **(G1)**, the DNA synthesis phase **(S)**, the second gap phase **(G2)**, and finally mitosis **(M) (Figure 11)** [71]. Quiescent cells in the G0 phase have dropped out of the cell-cycle [71]. In a model of cecal ligation and puncture-induced sepsis, Yang et al. showed that G1/S cell-cycle arrest was associated with AKI, and that recovery from AKI paralleled cell-cycle progression [72]. During cellular insults this G1/S cell-cycle arrest reduces oxygen consumption and prevents mitosis in damaged cells, thereby protecting the kidney from extended injuries. Cell-cycle arrest appears to protect cells from mitotic catastrophe, an event in which a cell becomes arrested in mitosis if chromosomal defects or problems affecting the mitotic machinery are sensed during the M phase [73]. Mitotic catastrophe can be defined as an oncosuppressive mechanism that precedes and is distinct from, yet operates through, either RCD or senescence [73].

The activities of complexes of cyclins and cyclin-dependent kinases **(CDK)**, which phosphorylate retinoblastoma **(RB)** family 'pocket proteins', drive progression through the cell-cycle **(Figure 11)** [71]. The ability of RB to sequester members of the E2F transcription-factor family, which allow for the expression of a transcriptional programme that enables progression through S-phase and mitosis, is inhibited by this phosphorylation [71]. Two classes of CDK inhibitors **(CDKI)**, which have differing mechanisms of action, modulate the activities of cyclin-CDK complexes **(Figure 11)**. The INK4 CDKI family (p15, p16, p18 and p19) inhibits CDK activity through direct interaction with and sequestration of the CDK concerned [71]. However, the

'CIP/KIP' CDKI family (p21, p27 and p57) binds to cyclin-CDK complexes. At high levels, p21 and p27 function as cyclin-E-CDK2 inhibitors, while at lower levels they promote the assembly of cyclin-D-CDK4 and cyclin-D-CDK6 complexes [71]. Both these complexes also function as a sink for p21 and p27, thereby relieving cyclin-E-CDK2 complexes from the p21/p27-mediated inhibition **(Figure 11)**. The regulatory pathways involved in the G1/S checkpoint are thus multiple, complex and interdependent [74]. Whether a cell will progress through the G1 phase of the cell-cycle is therefore determined by the relative levels of cyclins, CDKs and CDKIs [71]. Both cyclin-D- and cyclin-E-associated CDKs are believed to be required for G1-phase progression.

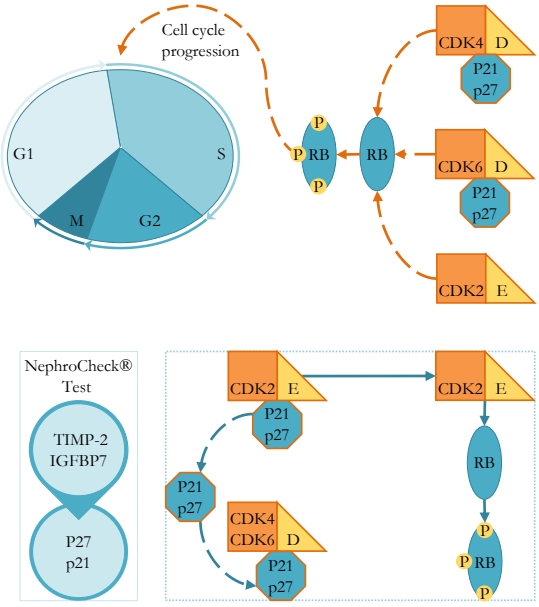


Figure 11 | Modulation of the eukaryotic cell-cycle (based on [71]; created with Motifolio)
Movement through the cell cycle is driven by the activities of complexes of cyclins and CDKs, which phosphorylate RB-family 'pocket proteins', thereby blocking their growth-inhibitory functions and permitting cell-cycle progression. Cyclin-D-CDK4 and cyclin-D-CDK6 complexes are active when associated with p21 or p27 CDKIs, but only cyclin-E-CDK2 complexes that are free of CDKIs are active *in vivo*.
Right box: Cyclin-D-CDK4 and cyclin-D-CDK6 complexes also function as a sink for p21 and p27, thereby relieving cyclin-E-CDK2 complexes from the p21/p27-mediated inhibition.
Left box: The NephroCheck® Test is an *in vitro* diagnostic device that quantitatively measures TIMP-2 and IGFBP7 proteins in human urine. In response to renal stress or damage, TIMP-2 and IGFBP7 are expressed in the kidney tubular epithelial cells of the proximal tubule. TIMP-2 stimulates p27 expression, while IGFBP7 increases the expression of p21. These effects are conducted in an autocrine and paracrine manner via TIMP-2 and IGFBP7 receptors.
Abbreviations: CDK cyclin-dependent kinase, CDKI CDK inhibitor, G1 first gap phase, G2 second gap phase, IGFBP7 insulin-like growth factor-binding protein 7, M mitosis, RB retinoblastoma, S DNA synthesis phase, TIMP-2 tissue inhibitor of metalloproteinases-2

III.A.3. Cellular suicide of kidney tubular epithelial cells

Non-apoptotic RCD pathways have gained special attention particularly due to their role in inflammation. The concept of immunogenic RCD suggests an auto-amplification loop: the release of DAMPs upon regulated necrosis (receptor-interacting protein kinase 3[21] **(RIPK3)** dependent or independent [73, 75, 76]) and ferroptosis amplifies the pro-inflammatory signal [75]. A unique feature of pyroptosis, a RCD pathway originally thought to occur exclusively in macrophages [75], is the maturation of the pro-inflammatory cytokines IL-1β and IL-18 during the RCD process, which depends on cleavage mediated by non-apoptotic caspase-1[22] [73]. Therefore, pyroptosis represents maximal immunogenicity [75].

The mode of RCD of kidney tubular epithelial cells in AKI has been a matter of intense debate. In IRI models, the protection from AKI reported for RIPK3-knockout mice likely involves vascular effects. Linkermann et al. showed that RIPK3-knockout mice exhibited statistically significant increases in the diameter of peritubular capillaries compared with wildtype mice, suggesting non-cell death functions for RIPK3 [77]. Further, they found that ferroptosis, a RCD pathway driven by loss of activity of the lipid repair enzyme glutathione peroxidase 4[23] **(GPX4)** and subsequent accumulation of lipid-based ROS (particularly lipid hydroperoxides) [78], mediated RCD of kidney tubular epithelial cells upon IRI [77]. Because RIPK3-knockout mice have been demonstrated to be resistant to AKI in sepsis models, the authors investigated first-generation ferrostatin (termed ferrostatin-1) – a ferroptosis inhibitor whose activity depends on the primary aromatic amine, which specifically inhibits accumulation of ROS from lipid oxidation [79] – in a model of lipopolysaccharides **(LPS)** *(Escherichia coli O111:B4 LPS)* induced shock, but no difference compared with vehicle-treated mice was evident [77].

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[21] uniprot/Q9Y572

[22] uniprot/P29466

[23] uniprot/P36969

III.B. Novel 'acute kidney stress' biomarkers

III.B.1. Acute kidney stress

Only recently Katz and Ronco proposed to describe the pre-phase that leads to AKI as 'acute kidney stress' **(AKS)** [80]. Whether AKS is a condition of increased susceptibility to exposures that lead to AKI (i.e., renal stress) or a condition of very early injury (i.e., renal damage), is debatable **(Figure 12)**. Regardless, biomarkers – or "measurable and quantifiable biological parameters" [81] – that respond to AKS open new horizons in regard to the early detection – or 'prediction' – of emerging AKI. These renal stress or damage biomarkers serve as surrogate measurements, estimating renal cell perfusion, function or metabolism [80].

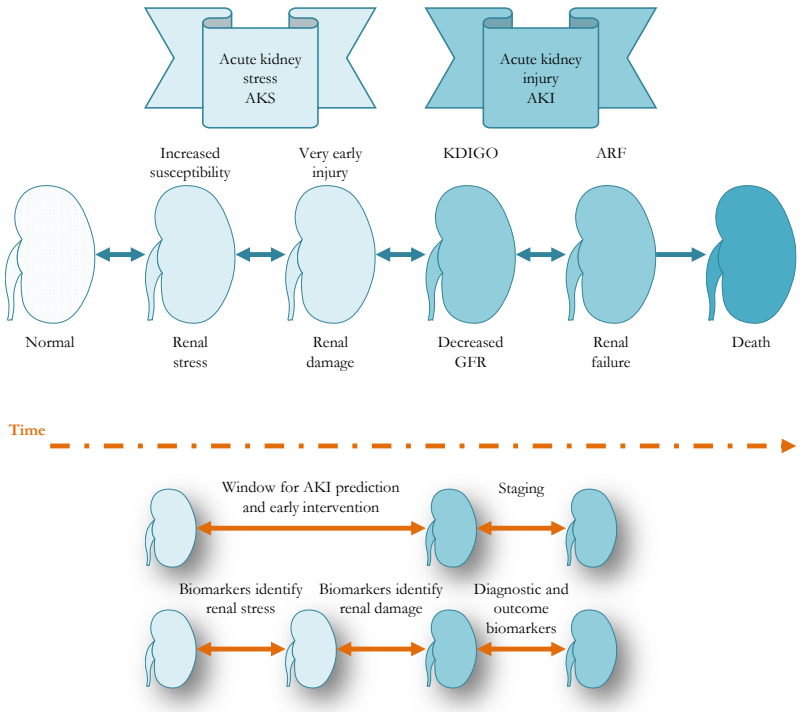


Figure 12 | Potential utilization of biomarkers for acute kidney injury (based on [82]; created with Motifolio)
A window of opportunity in which renal stress or damage has been initiated, but has not progressed to renal functional change, may be identified by assessment of AKS biomarkers.
Abbreviations: ARF acute renal failure, GFR glomerular filtration rate, KDIGO Kidney Disease | Improving Global Outcomes

In response to the urgent need for biomarkers that predict human AKI and ideally localize renal stress or damage to a specific nephron site, several promising urinary 'renal stress or damage biomarker candidates' have recently emerged **(Table 7)** [83]. These include, inter alia, neutrophil gelatinase-associated lipocalin **(NGAL) (Table 7)**. This dissertation will focus on NGAL and the in 2012 by our group discovered novel candidate chitinase 3-like protein 1 **(CHI3L1)** [84], which may appear and be measured in the urine of humans.

Table 7 | Nephron segment-specific urinary biomarkers of kidney injury (based on [83])

Glomerulus	Proximal tubules	Loop of Henle	Distal tubules	Collecting duct
Total protein	Kim-1	Osteopontin	Osteopontin	Calbindin D28
Cystatin C (urinary)	Clusterin	NHE-3	Clusterin	
β2-microglobulin	NGAL		GST-μ/π	
α1-microglobulin	GST-α		NGAL	
Albumin	β2-microglobulin		H-FABP	
	α1-microglobulin		Calbindin D28	
	NAG			
	Osteopontin			
	Cystatin C (urinary)			
	Netrin-1			
	RBP			
	IL-18			
	HGF			
	Cyr61			
	NHE-3			
	Exosomal fetuin-A			
	L-FABP			
	Albumin			

Abbreviations: Cyr cysteine-rich protein, GST glutathione S-transferase, H-FABP heart-type fatty acid-binding protein, HGF hepatocyte growth factor, IL-18 interleukin-18, Kim-1 kidney injury molecule-1, L-FABP liver-type fatty acid-binding protein, NAG N-acetyl-β-glucosaminidase, NGAL neutrophil gelatinase-associated lipocalin, NHE-3 sodium/hydrogen exchanger isoform 3, RBP retinol-binding protein

III.B.2. Neutrophil gelatinase-associated lipocalin

III.B.2.i. The lipocalin-family of proteins

Human NGAL[24] (also known as siderocalin, lipocalin-2 and human neutrophil lipocalin) is a member of the lipocalin-family of proteins [85]. The name 'lipocalin' reflects their ability to complex lipophilic molecules inside a cup-shaped protein fold [85]. The ligand pocket of NGAL can accommodate a spectrum of catecholic and phenolic iron chelators, but also iron-complexed

[24] uniprot/P80188

L-norepinephrine [85]. In keeping with the size range of lipocalins, the 178 amino acid deglycosylated human NGAL protein has a calculated molecular mass of 20,542 Dalton **(Da)** [86]. While N-glycosylation occurs at asparagine at position 65, cysteine at position 87 is responsible for dimerization [86]. A 3D protein structure model of human NGAL is shown in **Figure 13**.

Figure 13 | 3D protein structure model of human neutrophil gelatinase-associated lipocalin (adapted from the Research Collaboratory for Structural Bioinformatics Protein Data Bank; *viewer: JSmol; structure: 1NGL [87]; style: cartoon; display mode: secondary structure*)
The direction of the protein chain can be identified by arrows at the ends of β-sheets.

As suggested by its name, human NGAL is released from neutrophils as a disulphide-linked heterodimer with MMP-9[25] (also known as gelatinase B) [88]. However, it can be released from the secondary granules of neutrophils in two other forms as well: i.e., a monomer and a disulphide-linked homodimer [88]. Moreover, it is also markedly induced in injured epithelial cells, including the thick ascending limb and collecting duct cells of the kidney [89]. This suggests a potential involvement of NGAL in enhancing the epithelial phenotype – a documented effect of NGAL – both during kidney development and following AKI [90]. Finally, NGAL is markedly induced in a number of human cancers, where it promotes cancer progression through MMP-9 binding, thereby preventing MMP-9 degradation [91].

[25] uniprot/P14780

III.B.2.ii. Kidney iron transport: role of neutrophil gelatinase-associated lipocalin

The hepatic hormone hepcidin regulates iron metabolism systemically through its interaction with the iron exporter ferroportin-1[26] **(Fp-1)** on the basolateral membrane of enterocytes and the plasma membrane of macrophages [92]. Hepcidin binds to Fp-1, leading to Fp-1 endocytosis and degradation. Intracellular levels of iron are controlled by iron regulatory proteins that post-transcriptionally regulate the translation of iron metabolism proteins, including transferrin receptor protein 1[27] **(TfR1)** (promotion of translation when small iron pool), divalent metal transporter 1[28] **(DMT-1)** (promotion of translation when small iron pool), ferritin[29] (inhibition of translation when small iron pool) and Fp-1 (inhibition of translation when small iron pool) [92].

Kidney iron transport is illustrated in **Figure 14** [92]. The reader is encouraged to study this Figure together with the following paragraphs.

Circulating iron exists in the form of ferric iron **(Fe^{3+})**, either free or bound to, inter alia, transferrin[30] **(Tf)**, lactoferrin[31] **(LTf)** and NGAL. Through the membrane proteins DMT-1, TfR1, cubilin[32], megalin and NGAL receptor[33] **(NGALR)**, Fe^{3+} is imported into the kidney tubular epithelial cell, where it locates in the endosome. There, Fe^{3+} is reduced to ferrous iron **(Fe^{2+})** by the metalloreductase STEAP3[34] with ferrireductase activity. Finally, Fe^{2+} is released

[26] uniprot/Q9NP59

[27] uniprot/P02786

[28] uniprot/P49281

[29] uniprot/P02792 and uniprot/P02794

[30] uniprot/P02787

[31] uniprot/P02788

[32] uniprot/O60494

[33] uniprot/Q8WUG5

[34] uniprot/Q658P3

from the endosome into a labile iron pool in the cytoplasm through DMT-1. Excess iron is stored in the form of Fe^{2+} in ferritin, an iron storage protein complex [92].

Circulating iron bound to haem-(carrier proteins) exists in the form of Fe^{2+}. Through the membrane proteins haem carrier protein 1[35] **(HCP1)**, cubilin and megalin, haem-(carrier proteins) are imported into the cell. Inducible HO-1 catalyses the first and rate-limiting step in the degradation of haem, yielding biliverdin, carbon monoxide **(CO)** and Fe^{2+}. HO-1 can also upregulate the expression of ferritin. This coupled response thus degrades haem, generates antioxidants (i.e., bile pigments) and chelates iron. Finally, hephaestin[36] oxidizes Fe^{2+} to Fe^{3+}, while Fp-1 mediates Fe^{3+} export. Iron can also be exported into the interstitial fluid and circulation bound to haem through feline leukaemia virus subgroup C receptor[37] **(FLVCR)**. This also requires the extracellular presence of a suitable haem-binding protein (preferably haemopexin[38] **(HPX)**) [92]. As potential producers of HPX and the haemoglobin-binding protein haptoglobin[39], and expressers of the HPX receptor LRP-1[40] and the haemoglobin-haptoglobin complex receptor CD163[41], kidney macrophages help regulate renal iron metabolism through LRP-1- and CD163-mediated clearance in macrophages [92]. Likewise, renal tubular cells can secrete hepcidin and NGAL, which can possibly detoxify iron [92].

[35] uniprot/Q96NT5

[36] uniprot/Q9BQS7

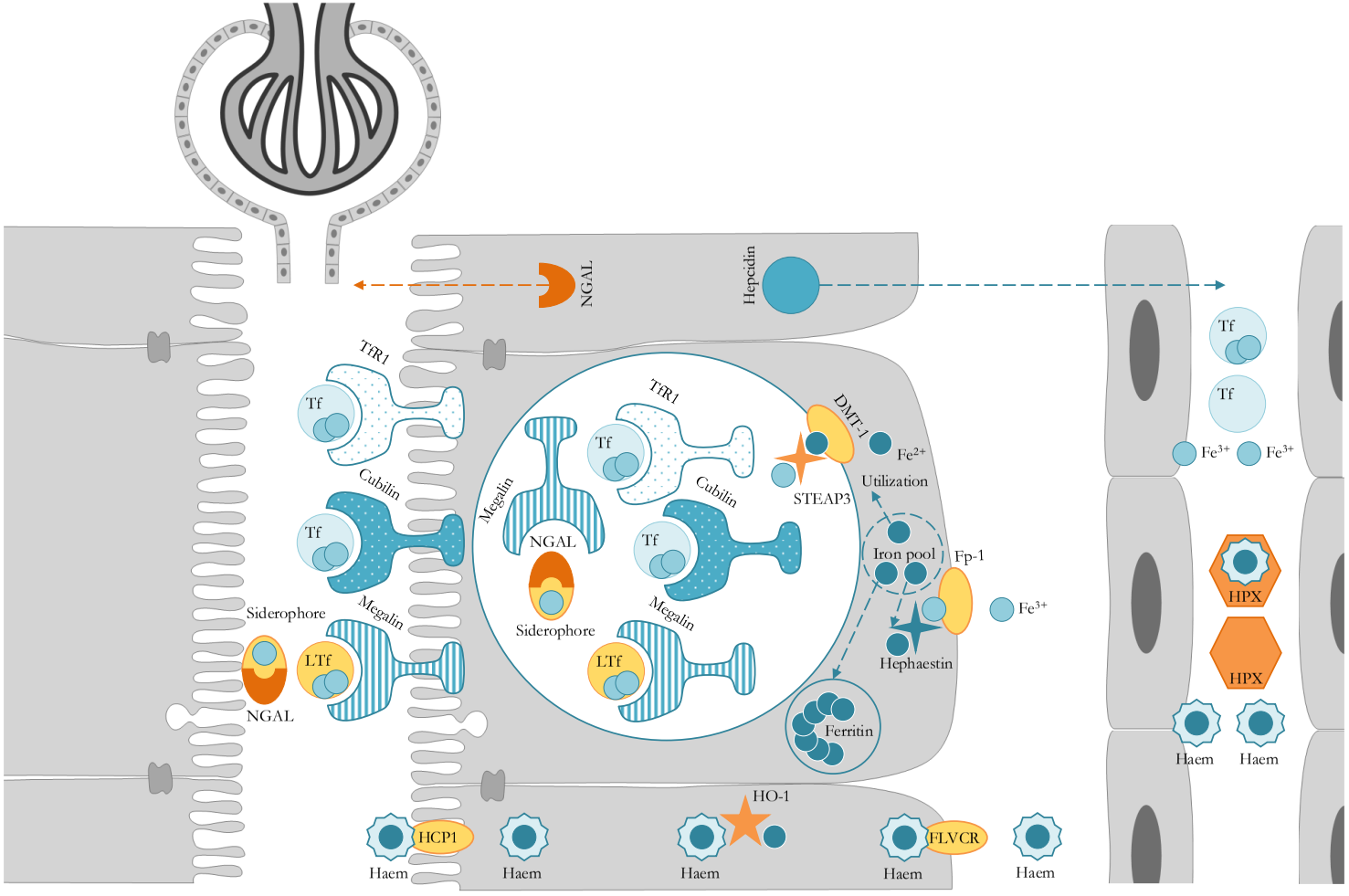
[37] uniprot/Q9Y5Y0

[38] uniprot/P02790

[39] uniprot/P00738

[40] uniprot/Q07954

[41] uniprot/Q86VB7


NGAL
Hepcidin
TfR1
Tf
Cubilin
Megalin
Siderophore
LTf
NGAL
DMT-1
Fe^{2+}
STEAP3
Utilization
Iron pool
Fp-1
Fe^{3+}
Hephaestin
Ferritin
HO-1
HCP1
FLVCR
Haem
Fe^{3+}
HPX

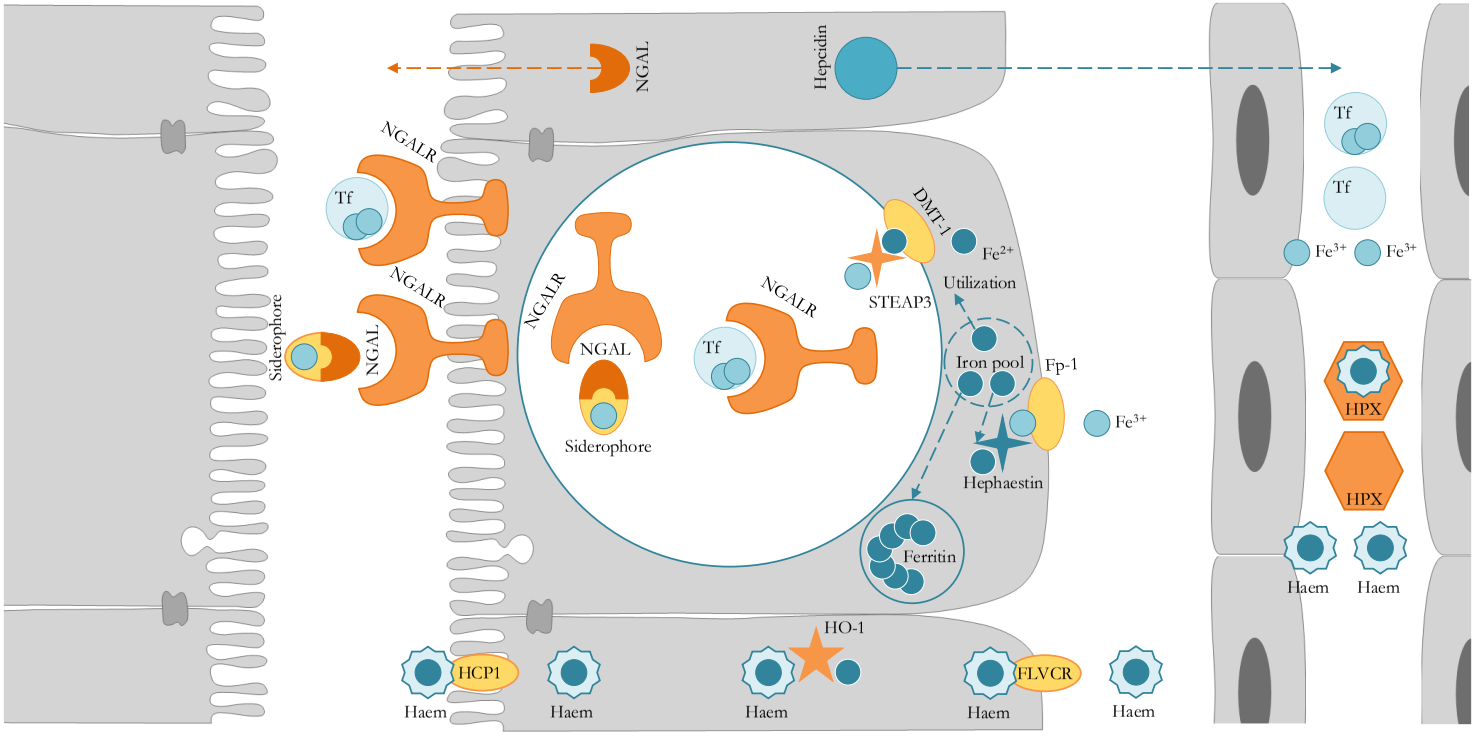


Figure 14 | Kidney iron transport in the proximal *(upper panel)* and distal *(lower panel)* tubule (based on [92]; created with Motifolio)
Note: megalin and cubilin also mediate uptake of haemoglobin and myoglobin for catabolism. Megalin facilitates endocytosis of cubilin.
Abbreviations: DMT-1 divalent metal transporter 1, Fe^{2+} ferrous iron, Fe^{3+} ferric iron, FLVCR feline leukaemia virus subgroup C receptor, Fp-1 ferroportin-1, HCP1 haem carrier protein 1, HO-1 haem oxygenase 1, HPX haemopexin, LTf lactoferrin, NGAL neutrophil gelatinase-associated lipocalin, NGALR neutrophil gelatinase-associated lipocalin receptor, STEAP3 metalloreductase, Tf transferrin, TfR1 transferrin receptor protein 1

III.B.3. Chitinase 3-like protein 1

III.B.3.i. The number-18 glycoside hydrolase-family of proteins

Glycoside hydrolases – also referred to as glycosidases and sometimes also as glycosyl hydrolases – are "enzymes that catalyse the hydrolysis of the glycosidic linkage of glycosides" [93]. The Carbohydrate-Active Enzymes database **(CAZy)** further classifies glycoside hydrolases in families based on amino acid sequence similarities [93]. The number-18 glycoside hydrolase-family is special in that it comprises catalytically inactive proteins such as chitinase-like proteins **(CLP)** in addition to catalytically active proteins such as chitinases [93]. These CLPs function as lectins because they can bind, but not hydrolyse, the glycan chitin, and therefore represent the 'chi-lectin' subfamily [94, 95].

Chitin is a linear homo-β-(1,4)-polymer made up of repeating units of β-N-acetyl-D-glucosamine [96]. Being the structural component in the coating of many living species including the cell wall of fungi, the cuticle of nematodes and the cysts of protozoan parasites, chitin is the second most abundant polysaccharide in nature after cellulose [96, 97]. In mammals, despite the absence of endogenous chitin, a number of chitinases and CLPs have been identified [97]. The 362 amino acid deglycosylated human CHI3L1[42] protein (also known as 39 kDa synovial protein, cartilage glycoprotein 39 and YKL-40 based on its three N-terminal amino acids (i.e., tyrosine Y, lysine K and leucine L)) has a calculated molecular mass of 40,476 Da [98, 99]. N-glycosylation occurs at asparagine at position 60 [100]. A 3D protein structure model of human CHI3L1 is shown in **Figure 15**.

[42] uniprot/P36222

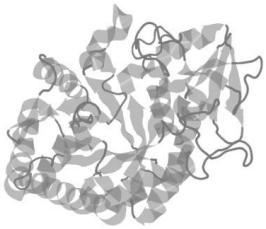


Figure 15 | 3D protein structure model of human chitinase 3-like protein 1 (adapted from the Research Collaboratory for Structural Bioinformatics Protein Data Bank; *viewer: JSmol; structure: 1NWR [101]; style: cartoon; display mode: secondary structure*)
The direction of the protein chain can be identified by arrows at the ends of β-sheets.

Although originally discovered as a secretion product of human synovial cells and osteoblasts [102, 103], it has become clear that a variety of cells including macrophages, neutrophils and kidney tubular epithelial cells can secrete CHI3L1 [104]. Regarding its function, it has become evident that CHI3L1 is enhanced in differentiated and polarized macrophages. Therefore, it has pleiotropic effects in inflammation [96]. Concentrations of either circulating or tissue CHI3L1 have been associated with a variety of inflammatory, remodelling, and neoplastic disorders, and with various primary and metastatic cancers [97].

III.B.3.ii. Background: galectin-glycan interactions, lipid rafts and signal transduction

Endogenous glycan-binding proteins or lectins "can specifically decode saccharide structures and glycosidic linkages and convey this structural information into functional cellular responses [105]." Some of these lectins effectively function as PRRs to initiate host innate immune responses [106]. One family of lectins is the galectin family, which is special in that it comprises soluble instead of transmembrane proteins **(Figure 16)** [107]. Most galectins are either bivalent or multivalent with regard to their carbohydrate-binding activities, enabling the formation of a spatial array of multivalent galectins and multivalent glycans – often termed the galectin-glycan lattice – on the cell surface **(Figure 16)** [107].

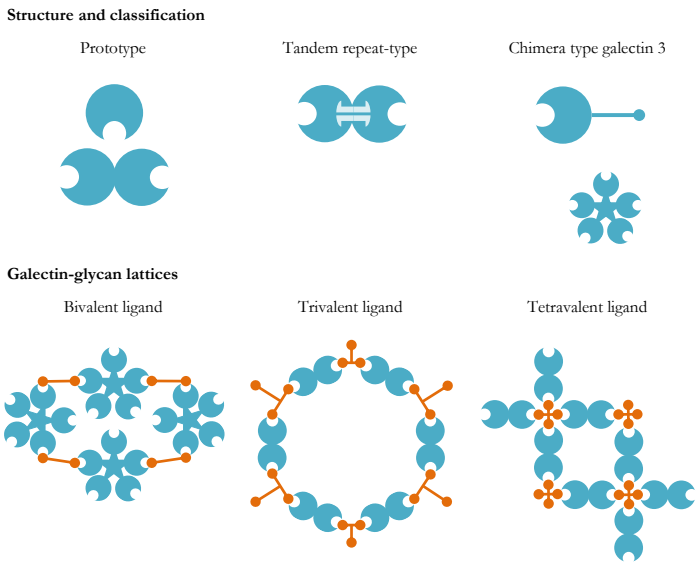


Figure 16 | Galectin-glycan interactions: structural features (based on [107]; created with Motifolio) Galectins can be subdivided into three groups: prototype galectins, which contain one CRD and can form homodimers; tandem repeat-type galectins, which contain two distinct CRDs in tandem and are inherently bivalent; and the chimera-type galectin 3, which contains a CRD connected to a non-lectin region. Distinct types of galectin-glycan lattices could be formed between multivalent galectins and multivalent glycans.
Abbreviations: CRD carbohydrate recognition domain

Initiation of signal transduction involves complex protein-protein interactions between inter alia extracellular ligands, transmembrane receptors and kinases, but lipid micro-environments on the same cell surface – also known as lipid rafts – take part in this process too **(Figure 17)** [108]. More specifically, galectin-glycan lattices could initiate signalling through one or more of such lipid rafts, in which the galectin-glycan lattice serves either as receptor-oligomerizing agent **(Figure 17, Model 1)** or as raft-clustering agent **(Figure 17, Model 2)** [105, 108].

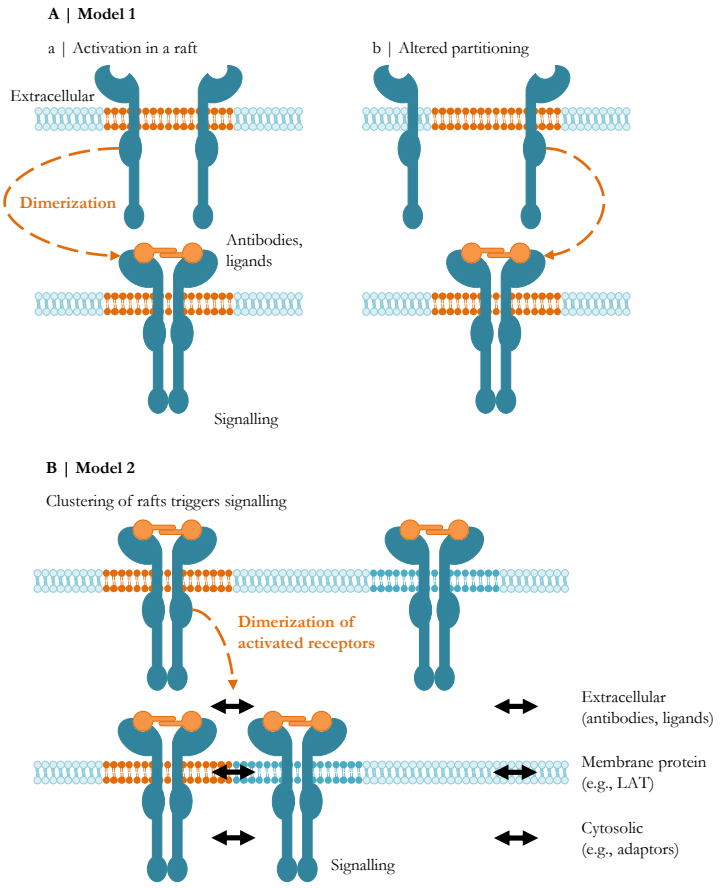


Figure 17 | Models of possible initiation of signal transduction through one or more lipid rafts (based on [108]; created with Motifolio)
Receptors could behave in at least three different ways in rafts. First, receptors associated at steady state with lipid rafts could be oligomerized and activated through either multivalent antibody (e.g., Fc receptor activated by IgE) or multivalent ligand (e.g., TCR activated by peptide presented on MHC protein) binding (A, a). Second, individual receptors with weak raft affinity could oligomerize upon either multivalent antibody or multivalent ligand binding, and this would lead to an increased residency time in rafts and receptor activation (A, b). Third, activated receptors could recruit crosslinking proteins (e.g., activated TCR recruits LAT) that bind to proteins in other rafts, and this would result in raft coalescence (B). Clustering could occur either extracellularly, within the membrane, or in the cytosol.
Abbreviations: Ig immunoglobulin, LAT linker for activation of T cells, MHC major histocompatibility complex, TCR T-cell antigen receptor

Surprisingly, only in 2013 He et al. investigated the possibility that the 'chi-lectin' subfamily member CHI3L1 mediates its biological effects through receptor binding, and identified at first IL-13 receptor α2[43] **(IL-13Rα2)** as its binding partner [109]. They hypothesised that CHI3L1(chi-

[43] uniprot/Q14627

lectin) and IL-13Rα2 (transmembrane protein) are part of a 'chi-lectin-glycan' lattice on the cell surface, including other lectins and glycoproteins [109].

III.B.3.iii. Inflammation signalling cascades: role of chitinase 3-like protein 1

Biological effects of CHI3L1 that are mediated through IL-13Rα2 binding include inhibition of apoptotic RCD in kidney tubular epithelial cells [109, 110], and inhibition of pyroptosis and IL-1β production in macrophages through inhibition of 'nucleotide-binding domain and leucine-rich repeat family pyrin domain containing 3'[44] **(NLRP3)** inflammasome activation [109, 111]. It is possible that the 'chi-lectin-glycan' lattice composed of inter alia CHI3L1 and IL-13Rα2 initiates signalling in inflammation signalling cascades through one or more lipid rafts. These inflammation signalling cascades may include the RAS-ERK and PI3K-mTOR pathway **(Figure 18 and Table 8)**. Indeed, He et al. demonstrated that the intracellular domain of IL-13Rα2 is not required for RAS-ERK and AKT activation [109]. Consequently, the authors postulated that IL-13Rα2 partners with another as of yet unidentified receptor to transduce CHI3L1 responses. This view is supported by previous studies showing that IL-13Rα2 co-immunoprecipitates with IL-4Rα[45] as component of the heterodimeric IL-13R that also includes IL-13Rα1[46] [112, 113] **(Figure 18 and Table 8)**. Initiation of either RAS-ERK or PI3K-mTOR signalling leads to inhibition of apoptotic RCD.

[44] uniprot/Q96P20

[45] uniprot/P24394

[46] uniprot/P78552

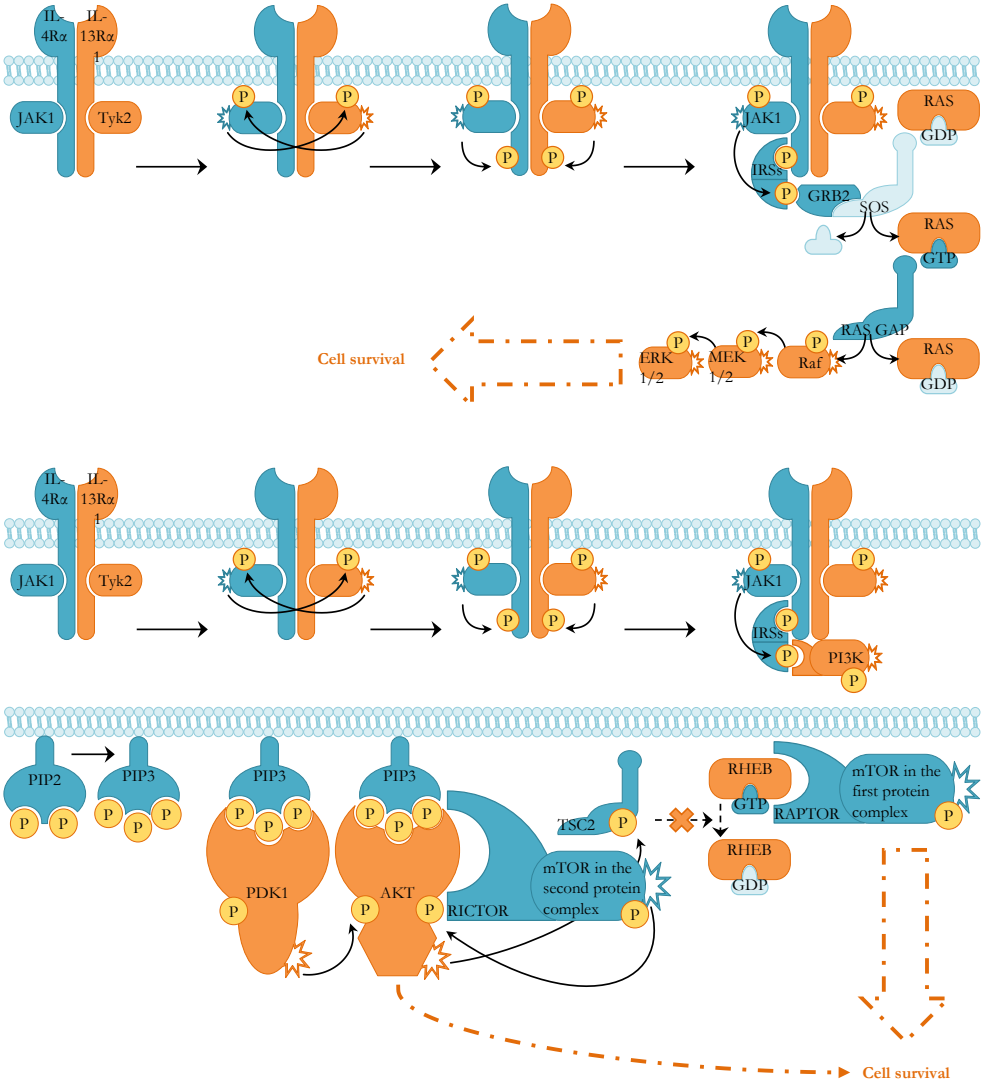


Figure 18 | The 'chi-lectin-glycan' lattice composed of inter alia CHI3L1 and IL-13Rα2 may initiate signalling in kidney tubular epithelial cells' inflammation signalling cascades through one or more lipid rafts (based on [114, 115]; created with Motifolio)
The *upper panel* shows the RAS-ERK pathway, while the *lower panel* shows the PI3K-mTOR pathway. The signal transducing partner of the 'chi-lectin-glycan' lattice could be the heterodimeric IL-13R that includes IL-4Rα and IL-13Rα1. Initiation of either RAS-ERK or PI3K-mTOR signalling leads to inhibition of apoptotic RCD in kidney tubular epithelial cells.
Table 8 *complements with detailed data lacking here for clarity purposes.*
Abbreviations: CHI3L1 chitinase 3-like protein 1, IL-13Rα2 interleukin-13 receptor α2, RCD regulated cell death

Likewise, it is possible that this 'chi-lectin-glycan' lattice hampers signalling in inflammation signalling cascades through one or more lipid rafts. These inflammation signalling cascades may include the TLR4-MYD88 pathway **(Figure 19 and Table 9)** as TLR4-MYD88 signalling is a prototypical priming event of NLRP3 inflammasome activation [116]. Hampering of TLR4-MYD88 signalling leads to inhibition of pyroptosis and IL-1β production in macrophages through inhibition of NLRP3 inflammasome activation.

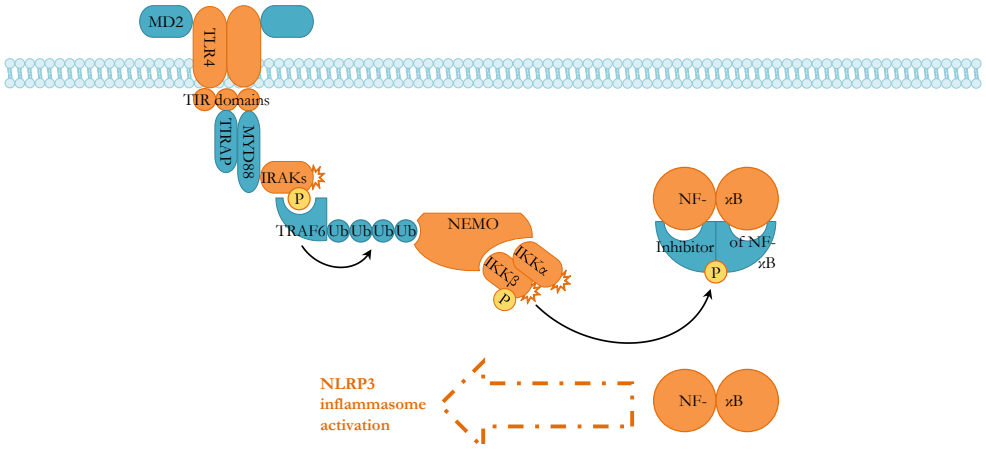


Figure 19 | The 'chi-lectin-glycan' lattice composed of inter alia CHI3L1 and IL-13Rα2 may hamper signalling in macrophages' inflammation signalling cascades through one or more lipid rafts (based on [116, 117]; created with Motifolio)
The TLR4-MYD88 pathway is shown. The signal transducing partner of the 'chi-lectin-glycan' lattice could be TLR4. Hampering of TLR4-MYD88 signalling leads to inhibition of pyroptosis and IL-1β production in macrophages through inhibition of NLRP3 inflammasome activation.
Table 9 *complements with detailed data lacking here for clarity purposes.*
Abbreviations: CHI3L1 chitinase 3-like protein 1, IL-13Rα2 interleukin-13 receptor α2, TLR4 Toll-like receptor 4

Table 8 | The RAS-ERK and the PI3K-mTOR pathways (based on [114, 115])

Type of signalling protein in RAS-ERK pathway	Name of signalling protein in RAS-ERK pathway
Receptor	Interleukin (IL)-13 receptor (R), which is comprised of IL-4Rα and IL-13Rα1
Tyrosine kinase	Janus tyrosine kinases (JAKs), i.e. JAK1 and Tyk2
Adaptor protein	Insulin receptor substrates (IRSs), i.e. IRS-1 and IRS-2, which are recruited to a specific tyrosine residue of IL-4Rα
Adaptor protein	GRB2
Guanine nucleotide exchange factor (GEF)	SOS
Guanosine triphosphate phosphohydrolase (GTPase)	Members of the RAS oncoprotein branch of the Ras family of proteins
GTPase-regulated kinase (MAPKKK)	Raf
Intermediate kinase (MAPKK)	MEK 1/2
Effector kinase (MAPK)	ERK 1/2

Type of signalling protein in PI3K-mTOR pathway	Name of signalling protein in PI3K-mTOR pathway
Receptor	Interleukin (IL)-13 receptor (R), which is comprised of IL-4Rα and IL-13Rα1
Tyrosine kinase	Janus tyrosine kinases (JAKs), i.e. JAK1 and Tyk2
Adaptor protein	Insulin receptor substrates (IRSs), i.e. IRS-1 and IRS-2, which are recruited to a specific tyrosine residue of IL-4Rα
Kinase	Phosphatidylinositol 3-kinase (PI3K)
Lipid messenger	Phosphatidylinositol 3,4,5 tri-phosphate (PIP3)
Kinase	AKT and PDK1, which recognize PIP3 and translocate to the membrane; mTOR in the second protein complex
GTPase activating protein (GAP)	TSC2
Guanosine triphosphate phosphohydrolase (GTPase)	RHEB
Kinase	mTOR in the first protein complex

GTPases have the intrinsic ability to catalyse the conversion of GTP (guanine triphosphate) to GDP (guanine diphosphate). GTPases are generally active when bound to GTP and inactive when bound to GDP. GEFs activate GTPases by inducing GTPases to release their bound GDP and thereby facilitating GTP binding. GAPs inactivate GTPases by inducing the GTPases to hydrolyse their bound GTP to GDP and become inactive.
PI3K can be activated upon phosphorylation by JAK1. PDK1 and mTOR in the second protein complex can be activated upon phosphorylation by PI3K.

Table 9 | The TLR4-MYD88 pathway (based on [116, 117])

Type of signalling protein in TLR4-MYD88 pathway	Name of signalling protein in TLR4-MYD88 pathway
Receptor and co-receptor	Toll-like receptor 4 (TLR4) and MD2
Adaptor protein	TIR domain-containing adaptor protein (TIRAP)
Adaptor protein	MYD88
Kinase (autophosphorylation?)	Interleukin (IL)-1 receptor-associated kinases (IRAKs)
Intermediate protein: ubiquitin ligase → K63-linked autoubiquitylation, which serves as signalling platform	TRAF6
Regulatory component of kinase	Nuclear factor (NF)-κB essential modifier (NEMO)
Kinase	Inhibitor of NF-κB kinase (IKK)

In most cells NF-κB exists in the cytoplasm as an inactive (heterodimeric) complex bound to inhibitor of NF-κB. Most agents that activate NF-κB do so through a common pathway based on phosphorylation-induced, proteasome-mediated degradation of inhibitor of NF-κB.
IKKβ is phosphorylated by an as of yet unidentified upstream kinase.

Chapter 2

Scientific Aim

Acute kidney injury represents an increasing global concern [118]. In response to the urgent need for biomarkers that predict human AKI and ideally localize renal stress or damage to a specific nephron site, our group discovered chitinase 3-like protein 3 **(CHI3L3)** as a novel candidate biomarker for sepsis-induced AKI by urinary proteomics [84, 119-121]. Validation with western blot analysis confirmed the presence of CHI3L3 in urine of septic mice with AKI, and its absence in urine of septic mice without AKI. In view of translational research [97], two other members of the number-18 glycoside hydrolase-family [93], i.e. CHI3L1 and acidic mammalian chitinase **(CHIA)**, showed similar results. Subsequently, we found that CHI3L1 measured in urine was more discriminative for the presence of AKI in human septic patients than CHIA [84], resulting in the filing of a patent application on this invention (the international patent application has been published as WO2012/136548).

The invention concerned relates to a method for evaluating renal status in a human subject, comprising: performing an assay method configured to detect CHI3L1 in a urine sample obtained from the subject to provide an assay result, i.e. a measured concentration of urinary CHI3L1 **(UCHI3L1)**; and correlating the assay result to the renal status of the subject. This dissertation focuses on one preferred embodiment of this invention, i.e. a method wherein the correlation step comprises assigning a diagnosis of the occurrence or non-occurrence of AKI to the subject based on the assay result.

For diagnostic AKI biomarker investigations, prospective cohort studies are optimal. Typically, a cardiac surgery cohort is chosen because these patients are homogeneous in terms of aetiology of AKI, and because the concentration-time-course of the novel biomarker after stress or damage to the kidneys can be easily monitored in these patients, as the timing of renal stress or damage is exactly known. AKI occurs in over half of general ICU patients [36], and sepsis patients (a condition that according to Reinhart et al. should be recognized as a global health priority too

[122]) are at particular risk for AKI [123], so the choice for additional clinical validation of UCHI3L1 in a heterogeneous ICU cohort is also evident.

Therefore, the **SCIENTIFIC AIM** of this dissertation was to provide **CLINICAL VALIDATION OF THE BIOMARKER UCHI3L1 AS EARLY DIAGNOSTIC OR 'PREDICTIVE' TOOL FOR EMERGING AKI IN SPECIFIC ADULT ICU SETTINGS**, comprising: **CORRELATING** a measured concentration of **CHI3L1 IN URINE TO KDIGO-BASED DIAGNOSIS** of the occurrence or non-occurrence of AKI; **AND COMPARING THIS PREDICTIVE ABILITY** with that of a measured concentration of SCr, a measured urine flow rate and a measured concentration of NGAL in urine, which is a well-known biomarker of acute tubular damage.

Chapter 3

Clinical Validation

of the Biomarker UCHI3L1
as Early Diagnostic or 'Predictive' Tool for Emerging AKI
in Adult ICU Patients
who either Underwent non-Cardiac Surgery
or Had a non-Surgical Reason for Critical Illness

A pilot study

Adapted from

DOI 10.1186/s13054-016-1192-x

De Loor J, Decruyenaere J, et al., (2016) Urinary chitinase 3-like protein 1 for early diagnosis of acute kidney injury: a prospective cohort study in adult critically ill patients. "Critical care: the official journal of the Critical Care Forum" DOI 10.1186/s13054-016-1192-x

I. Abstract

Background: AKI occurs frequently and adversely affects patient and kidney outcomes, especially when its severity increases from stage 1 to stages 2 or 3. Early interventions may counteract such deterioration, but this requires early detection. Our aim was to evaluate whether the novel renal damage biomarker UCHI3L1 can detect AKI stage ≥ 2 more early than SCr and UO, using the respective KDIGO criteria for definition and classification of AKI, and compare this to urinary NGAL **(UNGAL)**.

Methods: This was a translational single-center, prospective cohort study at the 22-bed surgical and 14-bed medical ICUs of Ghent University Hospital. We enrolled 181 severely ill adult patients who did not yet have AKI stage ≥ 2 based on the KDIGO criteria at time of enrolment. The concentration of Cr (serum, urine) and CHI3L1 (serum, urine) was measured at least daily, and UO hourly, in the period from enrolment till ICU discharge with a maximum of 7 ICU-days. The concentration of UNGAL was measured at enrolment. The primary endpoint was the development of AKI stage ≥ 2 within 12 h after enrolment.

Results: After enrolment, 21 (12 %) patients developed AKI stage ≥ 2 within the next 7 d, with 6 (3 %) of them reaching this condition within the first 12 h. The enrolment concentration of UCHI3L1 predicted the occurrence of AKI stage ≥ 2 within the next 12 h with a good area under the receiver-operating characteristics curve **(AUC-ROC)** of 0.792 (95 % CI: 0.726-0.849). This performance was similar to that of UNGAL (AUC-ROC = 0.748; 95 % CI: 0.678-0.810). Also, the samples collected in the 24-h time frame preceding diagnosis of the 1st episode of AKI stage ≥ 2 had a 2.0 times higher (95 % CI: 1.3-3.1) estimated marginal mean of UCHI3L1 than controls. We further found that increasing UCHI3L1 concentrations were associated with increasing AKI severity.

Conclusions: In this pilot study we found that UCHI3L1 was a good biomarker for prediction of AKI stage ≥ 2 in adult ICU patients.

II. Background

AKI occurs in approximately half of adult critically ill patients [35, 36, 38, 124-129]. Besides its recognized adverse effect on individual patient outcomes, both in the short- and long-term [35, 36, 38, 125-130], AKI causes an important socioeconomic burden ensuing from its relationship with the development of CKD [41], and ESRD requiring RRT [131].

Current diagnostic and staging criteria for AKI were defined by the KDIGO AKI work group and require monitoring of two surrogate GFR markers, i.e. SCr and UO, and of the intervention RRT [11]. As renal stress and damage to the kidneys precede the observed decline in GFR [63], diagnostic AKI biomarker research in the last decade has focused on detection of these early signals [121, 132, 133]. Studies have shown that urinary biomarkers like NGAL [134-138], and recently the panel tissue inhibitor of metalloproteinases-2 **(TIMP-2)** and insulin-like growth factor-binding protein 7 **(IGFBP7)** [136, 139, 140], can detect AKI in critically ill patients earlier than SCr or UO, even when using the most sensitive KDIGO criteria. In addition, these biomarkers may also allow detection of other outcomes such as progression of AKI, use of RRT, development of CKD, and long-term mortality [141, 142]. The complexity of the AKI syndrome and the interest in detecting other outcomes highly warrants evaluation of yet further candidate renal stress or damage biomarkers aiming to find either complementary or – less realistically – truly superior ones.

Recently, our group discovered CHI3L3 as a novel candidate biomarker for sepsis-induced AKI, by urinary proteomics [84, 119, 120]. Validation with western blot analysis confirmed the presence of CHI3L3 in urine of septic mice with AKI, and its absence in urine of septic mice without AKI. In view of translational research [97], two other members of the number-18 glycoside hydrolase-family [93], i.e. CHI3L1 and CHIA, showed similar results. Subsequently, we found that CHI3L1 measured in urine was more discriminative for the presence of AKI in human septic patients than CHIA [84].

The number-18 glycoside hydrolase-family is special in that it comprises catalytically inactive proteins such as CLPs (e.g., CHI3L1) in addition to catalytically active proteins such as chitinases [93]. These CLPs function as lectins because they can bind, but not hydrolyse, the glycan chitin, and therefore represent the chi-lectin subfamily [94, 95].

The objective of this study was first to evaluate the diagnostic performance of the urinary biomarker CHI3L1 for early detection of AKI stage ≥ 2 in adult critically ill patients, and then to compare this performance to that of NGAL, which was chosen as the reference urinary biomarker.

III. Methods *(see also section **VII.** "Supplemental Material to Methods")*

We followed recommendations for strengthening the reporting of observational studies in epidemiology **(STROBE)** *(Supplemental Table S1)* [143]. We will refer to AKI that was diagnosed and classified by KDIGO as $AKI_{SCr/UO}$, while AKI_{SCr} will imply that the KDIGO UO criteria were discarded *(Supplemental Table S2)*.

STUDY POPULATION

As a pilot study, we conducted a prospective cohort study at the 22-bed surgical and 14-bed medical ICUs of Ghent University Hospital from September 2012 till August 2014. The inclusion and exclusion criteria are shown in **Table 1**.

Table 1 | Inclusion and exclusion criteria of the study

Inclusion criteria	Exclusion criteria
Age ≥ 18 y	$AKI_{SCr/UO}$ stage ≥ 2 at time of enrolment[a]
Presence of both arterial and urinary catheter	CKD KDOQI stage 5 (GFR < 15 ml/min/1.73 m^2 or RRT) [144]
Expected ICU stay ≥ 48 h	
Respiratory SOFA score ≥ 2 (PaO_2/FiO_2 < 300) or cardiovascular SOFA score ≥ 1 (MAP < 70 mmHg or on vasopressor(s) for at least 1 h)	
Written informed consent	

[a]Based on the KDIGO criteria for AKI

Abbreviations: AKI acute kidney injury, CKD chronic kidney disease, FiO_2 fraction of inspired oxygen, GFR glomerular filtration rate, ICU intensive care unit, KDIGO Kidney Disease | Improving Global Outcomes, KDOQI Kidney Disease Outcomes Quality Initiative, MAP mean arterial pressure, PaO_2 partial pressure of oxygen in arterial blood, RRT renal replacement therapy, SCr serum creatinine, SOFA Sepsis-related Organ Failure Assessment, UO urine output

ETHICS, CONSENT AND PERMISSIONS

This study was approved by the Ethical Committee of Ghent University Hospital (Belgian registration number of the study: B670201213147), and conducted in accordance with the declaration of Helsinki and in compliance with the Good Clinical Practice Guidelines. All patients or their legally authorized representatives provided written informed consent.

SAMPLE COLLECTION, SAMPLE HANDLING, AND DATA COLLECTION

Blood and urine were collected at enrolment. The large majority of patients, i.e. 89 %, was enrolled on either the first (28 %) or second (61 %) ICU day, while the minority, i.e. 11 %, was enrolled on either the third (9 %) or fourth (2 %) ICU day. Each subject was sampled a second time on the day of enrolment **(d1)** at 6 pm if the first collection was before noon. The subsequent sampling times were at 6 am and 6 pm on d2-4, and at 6 am on d5-7 *(Supplemental Table S3A)*. This is similar to the methodology used in the hallmark study by Kashani et al. [136]. If the patient was discharged from the ICU before d7, the sampling stopped.

These paired blood and urine samples were collected by standard methods and centrifuged by standard protocols. Serum and urine supernatants were stored at -80°C and thawed at room temperature immediately prior to analysis. Clinical data needed to complete the individual clinical research files were extracted from the hospital records by study coordinators. Clinical data and samples were anonymized. *J. De Loor* had access to the anonymized SCr and serum C-reactive protein data in order to determine the appropriate sample dilution for CHI3L1 measurement by enzyme-linked immunosorbent assay **(ELISA)** *(Supplemental Table S3B)*. All other technicians were blinded to clinical data.

BIOMARKER MEASUREMENTS

Cr and UNGAL analyses were performed externally. Cr concentrations were measured with a kinetic rate-blanked Jaffé assay (commercial reagents, Roche Diagnostics, Basel, Switzerland) on a Cobas c502, while UNGAL concentrations were measured with a particle-enhanced turbidimetric

immunoassay **(PETIA)** (ST001-3CA, BioPorto, Hellerup, Denmark) on a Modular P. The concentration of CHI3L1 was determined in-house with a sandwich ELISA (DC3L10, R&D Systems, Minneapolis, MN, USA).

Both UCHI3L1 and UNGAL concentrations were statistically analysed as such and after correction for urine dilution by using the ratio to urinary creatinine **(UCr)**. The relative change in SCr measured at enrolment was defined as the ratio of the enrolment SCr to reference SCr. The UO after enrolment, defined as the mean UO in the first valid 6-h period after enrolment, was determined as the mean of the 6 UO values that were calculated each h in the first valid 6-h period after enrolment.

PRIMARY ENDPOINT

The primary endpoint of the study was the development of $AKI_{SCr/UO}$ stage ≥ 2 within 12 h after enrolment. Reference SCr was defined as the lowest SCr value within the last 3 mo prior to enrolment. The details for calculation of UO are outlined in the *Supplemental Material to Methods*.

SECONDARY ENDPOINTS

Secondary endpoints of the study were: $AKI_{SCr/UO}$ stage ≥ 2 within 24 h and 7 d after enrolment; AKI_{SCr} stage ≥ 2 within 12 h, 24 h and 7 d after enrolment.

UCHI3L1 RESPONSE TO AKI

We compared samples that were collected in the 24 h preceding diagnosis of the first episode of $AKI_{SCr/UO}$ stage ≥ 2 to those that were not followed by a first episode of $AKI_{SCr/UO}$ stage ≥ 2 within the next 24 h. For this analysis, we excluded all samples collected in the period starting from diagnosis of the first episode of $AKI_{SCr/UO}$ stage ≥ 2 till the end of the study.

For all 21 patients who developed $AKI_{SCr/UO}$ stage ≥ 2 (reference time 0 h) within 7 d after enrolment, we documented the UCHI3L1 concentrations corresponding with the time points 24 h before, 12 h before, 12 h after, and 24 h after diagnosis of the first episode of $AKI_{SCr/UO}$ stage ≥

2. This allowed us to investigate the distribution of UCHI3L1 over time in patients with $AKI_{SCr/UO}$ stage ≥ 2.

We also studied the distribution of UCHI3L1 in samples corresponding with different stages of severity of $AKI_{SCr/UO}$. If the total study period of 7 days was completed, 11 serum and 11 urine samples were available per ICU patient. All available UCHI3L1 concentrations were classified according to their $AKI_{SCr/UO}$ stage at that moment. As such, UCHI3L1 concentrations were divided into four groups: no $AKI_{SCr/UO}$ at the time of sampling, and $AKI_{SCr/UO}$ stages 1, 2, or 3 at the time of sampling.

STATISTICAL ANALYSIS

The primary analysis was based on comparison of the AUC-ROCs of UCHI3L1 with those of UNGAL for predicting the defined endpoints, which was performed in MedCalc 15.2.1 (MedCalc Software, Oostende, Belgium). We also calculated Spearman's coefficients of rank correlation with this program. In SPSS 22 (IBM, Armonk, NY, USA) we performed ***(1)*** mixed model analysis with log10(UCHI3L1) as the outcome variable; diagnosis of the first episode of $AKI_{SCr/UO}$ stage ≥ 2 within 24 h after sampling as the predictor variable; and patient as the random factor; ***(2)*** Fisher's exact or the chi-square test – the 95 % CI of a proportion was calculated with the Wilson procedure without correction for continuity [145, 146] – and the Mann-Whitney U test; ***(3)*** the Wilcoxon matched-pair signed-rank test; and ***(4)*** related-samples Friedman's two-way analysis of variance by ranks. Box and whisker plots were generated in GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). For all analyses, two-sided P values < 0.05 were considered statistically significant.

In the *Supplemental Material to Methods*, including *Supplemental Tables S3C-F*, we provide all details and also describe how the urinary biomarkers were introduced into the statistical programs.

IV. Results *(see also section **VIII.** "Supplemental Material to Results")*

PATIENT CHARACTERISTICS AND EVENT RATES

The patient flow diagram is presented in **Figure 1**.

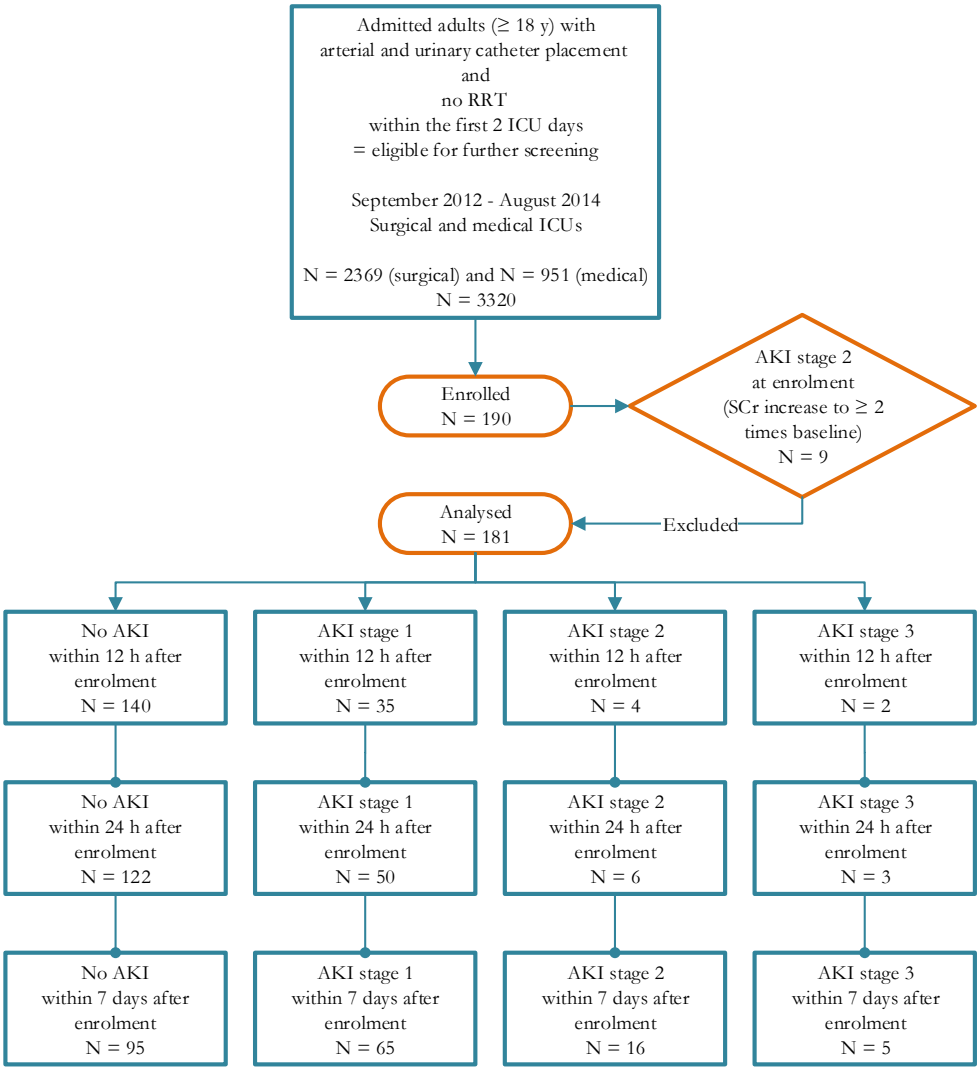


Figure 1 | Flow diagram of patient selection

AKI was defined and classified based on the KDIGO criteria.

Abbreviations: AKI acute kidney injury, ICU intensive care unit, KDIGO Kidney Disease | Improving Global Outcomes, RRT renal replacement therapy

Of the 190 enrolled patients in our study cohort, 9 already fulfilled the SCr criteria for AKI stage 2 at enrolment and were therefore excluded. In this analysis cohort (n = 181), 21 patients (12 %) developed $AKI_{SCr/UO}$ stage ≥ 2 within 7 d after enrolment. Within 24 h $AKI_{SCr/UO}$ stage ≥ 2 was met by 9 patients (5 %) and within 12 h by 6 patients (3 %). In **Table 2** the demographic information for these patients, either meeting or missing the primary endpoint, is depicted. Baseline characteristics were similar between both groups with the exception of an older age and a higher proportion of elective surgery in patients who developed $AKI_{SCr/UO}$ stage ≥ 2 within 12 h after enrolment.

Table 2 | Demographic information for patients of the analysis cohort

	Total	$AKI_{SCr/UO}$ stage ≥ 2[a]	No $AKI_{SCr/UO}$ stage ≥ 2[a]	P value
Number of patients	181 (100 %)	6 (3.3 %) [1.5-7.0 %]	175 (96.7 %) [93.0-98.5 %]	NA
Baseline characteristics				
Male sex	114 (63.0 %) [55.7-69.7 %]	4 (66.7 %) [30.0-90.3 %]	110 (62.9 %) [55.5-69.7 %]	1.000
Age (y)[b]	60.0 (51.0-70.0)	70.5 (65.8-78.0)	59.0 (50.0-70.0)	0.040
White race	181 (100 %) [97.9-100 %]	6 (100 %) [61.0-100 %]	175 (100 %) [97.9-100 %]	NA
BMI	24 (22-28)	25 (23-28)	24 (22-28)	0.594
Reference renal function				
SCr (mg/dl)	0.66 (0.52-0.79)	0.64 (0.55-0.69)	0.66 (0.51-0.79)	0.660
$eGFR_{CKD-EPI}$ (ml/min/1.73 m^2)	102 (89-118)	98 (92-102)	103 (89-118)	0.392
Diabetes mellitus				0.787
Type I	2 (1.1 %) [0.3-3.9 %]	0 (0.0 %) [0.0-39.0 %]	2 (1.1 %) [0.3-4.1 %]	
Type II	11 (6.1 %) [3.4-10.6 %]	0 (0.0 %) [0.0-39.0 %]	11 (6.3 %) [3.5-10.9 %]	
Heart failure				1.000
NYHA class I	179 (98.9 %) [96.1-99.7 %]	6 (100 %) [61.0-100 %]	173 (98.9 %) [95.9-99.7 %]	
NYHA class II	2 (1.1 %) [0.3-3.9 %]	0 (0.0 %) [0.0-39.0 %]	2 (1.1 %) [0.3-4.1 %]	
NYHA class III	0 (0.0 %) [0.0-2.1 %]	0 (0.0 %) [0.0-39.0 %]	0 (0.0 %) [0.0-2.1 %]	
NYHA class IV	0 (0.0 %) [0.0-2.1 %]	0 (0.0 %) [0.0-39.0 %]	0 (0.0 %) [0.0-2.1 %]	
Myocardial infarction or cardiac arrest[c]	17 (9.4 %) [5.9-14.5 %]	0 (0.0 %) [0.0-39.0 %]	17 (9.7 %) [6.2-15.0 %]	1.000
Malignancy[c]	17 (9.4 %) [5.9-14.5 %]	1 (16.7 %) [3.0-56.4 %]	16 (9.1 %) [5.7-14.3 %]	0.451
ICU admission				
Referred from				0.063
Other hospital	42 (23.2 %) [17.7-29.9 %]	0 (0.0 %) [0.0-39.0 %]	42 (24.0 %) [18.3-30.8 %]	

	Total	$AKI_{SCr/UO}$ stage ≥ 2[a]	No $AKI_{SCr/UO}$ stage ≥ 2[a]	P value
Emergency room	75 (41.4 %) [34.5-48.7 %]	1 (16.7 %) [3.0-56.4 %]	74 (42.3 %) [35.2-49.7 %]	
Operating room	21 (11.6 %) [7.7-17.1 %]	1 (16.7 %) [3.0-56.4 %]	20 (11.4 %) [35.8-50.3 %]	
Floor	43 (23.8 %) [18.1-30.5 %]	4 (66.7 %) [30.0-90.3 %]	39 (22.3 %) [16.8-29.0 %]	
Reason				0.038
Medical	108 (59.7 %) [52.4-66.5 %]	3 (50.0 %) [18.8-81.2 %]	105 (60.0 %) [52.6-67.0 %]	
Elective surgery	13 (7.2 %) [4.2-11.9 %]	2 (33.3 %) [9.7-70.0 %]	11 (6.3 %) [3.5-10.9 %]	
Urgent surgery	60 (33.1 %) [26.7-40.3 %]	1 (16.7 %) [3.0-56.4 %]	59 (33.7 %) [27.1-41.0 %]	
First day of study				
SOFA score (points)	9 (7-11)	11 (8-16)	9 (7-11)	0.189
Mechanical ventilation	122 (67.4 %) [60.3-73.8 %]	5 (83.3 %) [43.6-97.0 %]	117 (66.9 %) [59.6-73.4 %]	0.665
Vasopressors	117 (64.6 %) [57.4-71.2 %]	4 (66.7 %) [30.0-90.3 %]	113 (64.6 %) [57.2-71.3 %]	1.000
Infection	153 (84.5 %) [78.6-89.1 %]	6 (100 %) [61.0-100 %]	147 (84.0 %) [77.8-88.7 %]	0.592
Infection ++[d]	122 (67.4 %) [60.3-73.8 %]	6 (100 %) [61.0-100 %]	116 (66.3 %) [59.0-72.9 %]	0.179
Nephrotoxic drugs (before or at the first study day)				
ACE inhibitors	25 (13.8 %) [9.5-19.6 %]	1 (16.7 %) [3.0-56.4 %]	24 (13.7 %) [9.4-19.6 %]	0.596
ARBs	5 (2.8 %) [1.2-6.3 %]	0 (0.0 %) [0.0-39.0 %]	5 (2.9 %) [1.2-6.5 %]	1.000
Iodinated contrast media	81 (44.8 %) [37.7-52.0 %]	2 (33.3 %) [9.7-70.0 %]	79 (45.1 %) [38.0-52.5 %]	0.693
Aminoglycosides	6 (3.3 %) [1.5-7.0 %]	0 (0.0 %) [0.0-39.0 %]	6 (3.4 %) [1.6-7.3 %]	1.000
Vancomycin	15 (8.3 %) [5.1-13.2 %]	0 (0.0 %) [0.0-39.0 %]	15 (8.6 %) [5.3-13.7 %]	1.000
Diuretics	45 (24.9 %) [19.1-31.6 %]	3 (50.0 %) [18.8-81.2 %]	42 (24.0 %) [18.3-30.8 %]	0.164
Tacrolimus	2 (1.1 %) [0.3-3.9 %]	1 (16.7 %) [3.0-56.4 %]	1 (0.6 %) [0.1-3.2 %]	0.065
Cyclosporine	1 (0.6 %) [0.1-3.1 %]	0 (0.0 %) [0.0-39.0 %]	1 (0.6 %) [0.1-3.2 %]	1.000
NSAIDs (chronic)	10 (5.5 %) [3.0-9.9 %]	1 (16.7 %) [3.0-56.4 %]	9 (5.1 %) [2.7-9.5 %]	0.292
Corticosteroids (chronic)	24 (13.3 %) [9.1-19.0 %]	1 (16.7 %) [3.0-56.4 %]	23 (13.1 %) [8.9-18.9 %]	0.580

Data represent number (proportion) [95 % CI of proportion] for categorical variables and median (IQR) for continuous variables.

[a]Within 12 h after enrolment (primary endpoint); based on the KDIGO criteria for AKI

[b]Determined at the 1st day of the study

[c]At time of hospital or ICU admission

[d]Suspected bacterial infection, either leading to arterial hypotension or organ dysfunction, or leading to shock *(Supplemental Table S4)*

Abbreviations: ACE angiotensin converting enzyme, AKI acute kidney injury, ARB angiotensin-II receptor blocker, BMI body mass index, CI confidence interval, CKD-EPI Chronic Kidney Disease Epidemiology Collaboration, eGFR estimated glomerular filtration rate, ICU intensive care unit, IQR interquartile range, KDIGO Kidney Disease | Improving Global Outcomes, NSAID nonsteroidal anti-inflammatory drug, NYHA New York Heart Association, SCr serum creatinine, SOFA Sepsis-related Organ Failure Assessment, UO urine output

In **Table 3** the distribution of patients over different SCr and UO AKI stages that were maximally reached within 12 h, 24 h and 7 d after enrolment is shown.

Table 3 | Distribution of patients over different stages of severity of acute kidney injury maximally reached within indicated observation periods

Time window	Maximum AKI stage	UO 0[a]	UO 1[a]	UO 2[a]	UO 3[a]	Total in row
12 h	SCr 0[a]	140 (77 %)	11 (6 %)	2 (1 %)	0 (0 %)	153 (85 %)
24 h		122 (67 %)	19 (10 %)	4 (2 %)	0 (0 %)	145 (80 %)
7 days		95 (52 %)	23 (13 %)	2 (1 %)	0 (0 %)	120 (66 %)
12 h	SCr 1[a]	21 (12 %)	3 (2 %)	0 (0 %)	0 (0 %)	24 (13 %)
24 h		27 (15 %)	4 (2 %)	0 (0 %)	0 (0 %)	31 (17 %)
7 days		35 (19 %)	7 (4 %)	3 (2 %)	0 (0 %)	45 (25 %)
12 h	SCr 2[a]	1 (1 %)	1 (1 %)	0 (0 %)	0 (0 %)	2 (1 %)
24 h		0 (0 %)	2 (1 %)	0 (0 %)	0 (0 %)	2 (1 %)
7 days		4 (2 %)	4 (2 %)	3 (2 %)	0 (0 %)	11 (6 %)
12 h	SCr 3[a]	1 (1 %)	1 (1 %)	0 (0 %)	0 (0 %)	2 (1 %)
24 h		2 (1 %)	1 (1 %)	0 (0 %)	0 (0 %)	3 (2 %)
7 days		4 (2 %)	1 (1 %)	0 (0 %)	0 (0 %)	5 (3 %)
12 h	Total in column	163 (90 %)	16 (9 %)	2 (1 %)	0 (0 %)	181 (100 %)
24 h		151 (83 %)	26 (14 %)	4 (2 %)	0 (0 %)	181 (100 %)
7 days		138 (76 %)	35 (19 %)	8 (4 %)	0 (0 %)	181 (100 %)

Data represent number (proportion).
Patients with different severity stages of $AKI_{SCr/UO}$ maximally reached within indicated observation periods are marked with differently coloured kidneys:
represents AKI stage 1, represents AKI stage 2 and represents AKI stage 3.
[a]SCr 0 represents no AKI based on the $KDIGO_{SCr}$ criteria. Likewise, UO 0 represents no AKI based on the $KDIGO_{UO}$ criteria. SCr 1/2/3 represents AKI stage 1/2/3 based on the $KDIGO_{SCr}$ criteria. Likewise, UO 1/2/3 represents AKI stage 1/2/3 based on the $KDIGO_{UO}$ criteria.
Abbreviations: AKI acute kidney injury, KDIGO Kidney Disease | Improving Global Outcomes, SCr serum creatinine, UO urine output

BIOMARKERS' DIAGNOSTIC PERFORMANCES

The biomarkers UCHI3L1 and UNGAL, both measured at enrolment, were good predictors of the development of $AKI_{SCr/UO}$ stage ≥ 2 within the next 12 h, with an AUC-ROC of 0.792 (95 % CI: 0.726-0.849) for UCHI3L1 and 0.748 (95 % CI: 0.678-0.810) for UNGAL (P = 0.587) **(Figure 2)**. When UO criteria were discarded, these AUC-ROCs improved to 0.877 (95 % CI: 0.820-0.921) for UCHI3L1 and 0.865 (95 % CI: 0.807-0.911) for UNGAL (P = 0.661). Extending our prediction window to 24 h slightly decreased the AUC-ROC for both biomarkers for

predicting $AKI_{SCr/UO}$ stage ≥ 2, while the AUC-ROC for predicting AKI_{SCr} stage ≥ 2 remained similar **(Figure 2)**. In the 7-d prediction window both biomarkers became poor predictors of AKI stage ≥ 2, irrespective of the definition used. To obtain two explicit clinical phenotypes, the AUC-ROC analyses were repeated excluding AKI stage 1 (comparing AKI stage ≥ 2 with no AKI). These showed essentially unchanged results for UCHI3L1 and UNGAL, both measured at enrolment *(Supplemental Table S5)*.

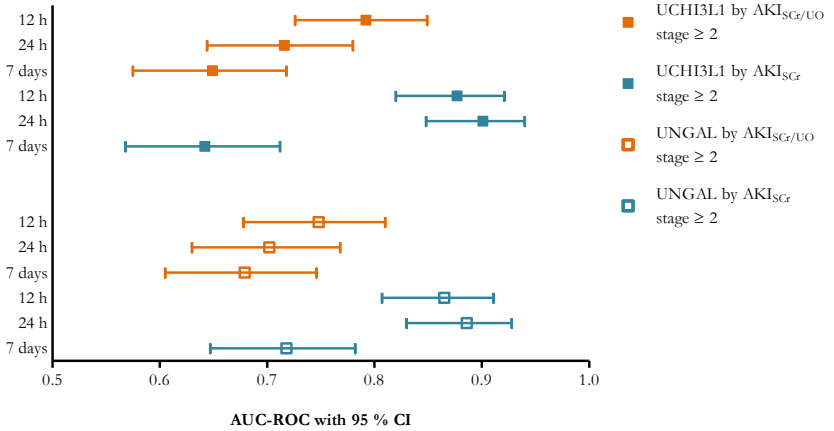


Figure 2 | Areas under receiver-operating characteristics curves of UCHI3L1 and UNGAL at enrolment for prediction of the six different endpoints
The P value for the difference in AUC-ROC between both biomarkers for predicting AKI stage ≥ 2 based on the KDIGO criteria ($AKI_{SCr/UO}$) within 12 h after enrolment was 0.587. In the 24-h prediction window the P value was 0.823, and in the 7-d prediction window it was 0.522. Likewise, the P value for the difference in AUC-ROC between both biomarkers for predicting AKI_{SCr} stage ≥ 2 within 12 h after enrolment was 0.661. In the 24-h prediction window the P value was 0.495, and in the 7-d prediction window it was 0.099.
Abbreviations: AKI acute kidney injury, AUC-ROC area under the receiver-operating characteristics curve, KDIGO Kidney Disease | Improving Global Outcomes, SCr serum creatinine, UCHI3L1 urinary chitinase 3-like protein 1, UNGAL urinary neutrophil gelatinase-associated lipocalin, UO urine output

Correction of the urinary biomarker concentrations for urine dilution by calculating the ratio to UCr, decreased the AUC-ROC for both biomarkers when diagnosing AKI stage ≥ 2 based on SCr or UO, while there was no difference observed when diagnosing AKI stage ≥ 2 based on SCr alone **(Table 4)**.

Table 4 | Comparison of areas under receiver-operating characteristics curves

Biomarker measurement	Time window	$AKI_{SCr/UO}$ stage ≥ 2[a] AUC-ROC	95 % CI	P value[c]	AKI_{SCr} stage ≥ 2[b] AUC-ROC	95 % CI	P value[c]
			N = 181				
Enrolment UCHI3L1	12 h	0.792	0.726-0.849		0.877	0.820-0.921	
	24 h	0.716	0.644-0.780		0.901	0.848-0.940	
Comparison with							
Enrolment UCr-corrected UCHI3L1	12 h	0.674	0.601-0.742	0.038	0.832	0.769-0.883	0.359
	24 h	0.587	0.511-0.659	0.005	0.861	0.802-0.908	0.308
Enrolment SCHI3L1	12 h	0.645	0.570-0.714	0.071	0.784	0.717-0.842	0.300
	24 h	0.548	0.472-0.622	0.022	0.830	0.767-0.881	0.324
Enrolment SCr	12 h	0.624	0.549-0.695	0.113	0.732	0.662-0.795	0.363
	24 h	0.639	0.565-0.709	0.406	0.766	0.697-0.826	0.278
Enrolment SCr to reference SCr ratio	12 h	0.690	0.618-0.757	0.274	0.806	0.741-0.861	0.614
	24 h	0.695	0.622-0.761	0.784	0.847	0.786-0.896	0.622
Enrolment UNGAL	12 h	0.748	0.678-0.810		0.865	0.807-0.911	
	24 h	0.702	0.630-0.768		0.886	0.830-0.928	
Comparison with							
Enrolment UCr-corrected UNGAL	12 h	0.622	0.547-0.693	0.007	0.795	0.729-0.851	0.123
	24 h	0.570	0.495-0.644	0.001	0.826	0.763-0.878	0.109
			N = 180[d]				
Enrolment UCHI3L1	12 h	0.791	0.724-0.848		0.876	0.819-0.921	
	24 h	0.715	0.643-0.779		0.901	0.847-0.940	
Comparison with							
UO after enrolment[e]	12 h	0.833	0.771-0.885	0.728	0.759	0.690-0.820	0.034
	24 h	0.808	0.743-0.863	0.438	0.795	0.728-0.851	0.022

[a]Based on the KDIGO criteria for AKI
[b]Based on the KDIGO SCr criteria for AKI
[c]The P value is shown for the difference in AUC-ROC. We always compared with the AUC-ROC of the urinary biomarker (UCHI3L1or UNGAL) marked in colour.
[d]UO after enrolment could not be calculated in 1 patient.
[e]Defined as the mean UO in the first valid 6-h period after enrolment (in ml/kg/h)
Abbreviations: AKI acute kidney injury, AUC-ROC area under the receiver-operating characteristics curve, CI confidence interval, KDIGO Kidney Disease | Improving Global Outcomes, SCHI3L1 serum chitinase 3-like protein 1, SCr serum creatinine, UCHI3L1 urinary chitinase 3-like protein 1, UCr urinary creatinine, UNGAL urinary neutrophil gelatinase-associated lipocalin, UO urine output

In contrast to UCHI3L1, serum CHI3L1 **(SCHI3L1)** measured at enrolment was a poor predictor of $AKI_{SCr/UO}$ stage ≥ 2 within the next 12 h, with an AUC-ROC of 0.645 (95 % CI: 0.570-0.714). In the 24-h prediction window SCHI3L1 did not predict $AKI_{SCr/UO}$ stage ≥ 2. Again, when diagnosing based on SCr alone, the AUC-ROC for SCHI3L1 improved, i.e. AUC-ROC of 0.784 (95 % CI: 0.717-0.842) in the 12-h and AUC-ROC of 0.830 (95 % CI: 0.767-0.881) in the 24-h prediction window **(Table 4)**.

We additionally studied the discriminatory ability of the individual KDIGO parameters, i.e. (relative change in) SCr measured at enrolment and UO after enrolment **(Table 4)**. The AUC-ROC was similar for UCHI3L1, SCr and UO. However, UCHI3L1 performed better than UO when diagnosing AKI stage ≥ 2 based on SCr alone (P = 0.034 in the 12-h prediction window; P = 0.022 in the 24-h prediction window). In addition, compared to SCr, there was a clear trend towards a better AUC-ROC for UCHI3L1, especially when diagnosing AKI stage ≥ 2 based on SCr or UO within 12 h after enrolment.

Combining both urinary biomarkers as the two-biomarker panel [UCHI3L1]•[UNGAL] did not improve the diagnostic performance of each of these single biomarkers for predicting either the 12-h or 24-h endpoints **(Table 5)**. A positive relationship between these markers was observed, with a Spearman's coefficient of rank correlation of 0.615 (95 % CI: 0.515-0.698) *(Supplemental Table S6)*.

With the Youden index a cutoff for UNGAL of 139 ng/ml was identified corresponding to the reference cutoff for this variable (> 150 ng/ml [135]) *(Supplemental Table S7)*.

Table 5 | Comparison of areas under receiver-operating characteristics curves between the two-biomarker panel [UCHI3L1]•[UNGAL] and each of these single biomarkers

		$AKI_{SCr/UO}$ stage ≥ 2[a]			AKI_{SCr} stage ≥ 2[b]		
	Time window	AUC-ROC	95 % CI	P value	AUC-ROC	95 % CI	P value
Enrolment [UCHI3L1]•[UNGAL]	12 h	0.784	0.717-0.841	0.764[c]	0.874	0.817-0.919	0.879[c]
				0.522[d]			0.544[d]
	24 h	0.721	0.650-0.785	0.856[c]	0.899	0.845-0.939	0.877[c]
				0.622[d]			0.311[d]

[a]Based on the KDIGO criteria for AKI
[b]Based on the KDIGO SCr criteria for AKI
[c]Difference in AUC-ROC between the two-biomarker panel and UCHI3L1, both measured at enrolment
[d]Difference in AUC-ROC between the two-biomarker panel and UNGAL, both measured at enrolment
Abbreviations: AKI acute kidney injury, AUC-ROC area under the receiver-operating characteristics curve, CI confidence interval, KDIGO Kidney Disease | Improving Global Outcomes, SCr serum creatinine, UCHI3L1 urinary chitinase 3-like protein 1, UNGAL urinary neutrophil gelatinase-associated lipocalin, UO urine output

UCHI3L1 RESPONSE TO AKI

Additions to the published paper are marked in italic font.

Samples collected in the 24 h preceding diagnosis of the first episode of $AKI_{SCr/UO}$ stage ≥ 2 had a 2.0 times higher (95 % CI: 1.3-3.1) estimated marginal mean of UCHI3L1 than those not followed by a first episode of $AKI_{SCr/UO}$ stage ≥ 2 within the next 24 h. The general time-concentration profile of UCHI3L1 showed a trend for increasing concentrations from 24 h before, towards diagnosis of the first episode of $AKI_{SCr/UO}$ stage ≥ 2 (reference time 0 h) **(Figure 3)**. After reference time 0 h, median UCHI3L1 concentrations showed a decreasing trend. Samples corresponding with $AKI_{SCr/UO}$ stage 1 at time of collection had higher UCHI3L1 concentrations than those corresponding with no $AKI_{SCr/UO}$ at time of collection ($P < 0.001$) **(Figure 4 *and Table 6*)**. Stage 1 and stage 2 samples had similar UCHI3L1 concentrations ($P = 0.514$). Stage 3 samples again had higher UCHI3L1 concentrations than stage 2 samples ($P < 0.001$). *The individual concentration-time-course of UCHI3L1 with the KDIGO stage-time-course are visually presented in **Figure 5** for two AKI patients.*

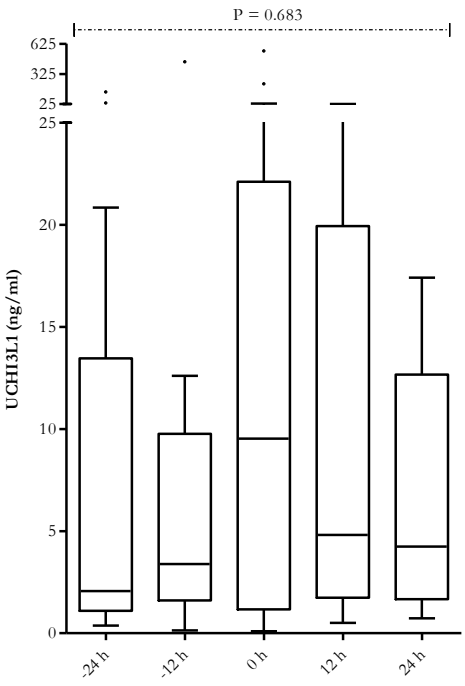


Figure 3 | Distribution of UCHI3L1 over time in patients who developed AKI stage ≥ 2 based on the KDIGO criteria ($AKI_{SCr/UO}$) within 7 d after enrolment (n = 21)

The reference time 0 h represents time of diagnosis of the first episode of $AKI_{SCr/UO}$ stage ≥ 2. At time -24 h, a UCHI3L1 concentration was available for 14 of the 21 patients. Likewise, 13 values were available at time -12 h, 19 at reference time 0 h, 14 at time 12 h, and 12 at time 24 h

Abbreviations: AKI acute kidney injury, KDIGO Kidney Disease | Improving Global Outcomes, SCr serum creatinine, UCHI3L1 urinary chitinase 3-like protein 1, UO urine output

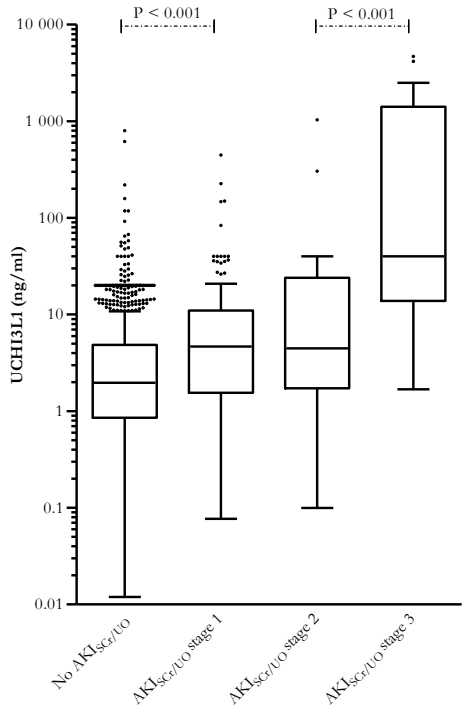


Figure 4 | Distribution of UCHI3L1 in samples corresponding with different stages of severity of AKI based on the KDIGO criteria ($AKI_{SCr/UO}$)

Abbreviations: AKI acute kidney injury, KDIGO Kidney Disease | Improving Global Outcomes, SCr serum creatinine, UCHI3L1 urinary chitinase 3-like protein 1, UO urine output

Table 6 | Quartiles of UCHI3L1 in samples corresponding with different stages of severity of AKI based on the KDIGO criteria ($AKI_{SCr/UO}$)

	No $AKI_{SCr/UO}$[a]	$AKI_{SCr/UO}$ stage 1[a]	$AKI_{SCr/UO}$ stage 2[a]	$AKI_{SCr/UO}$ stage 3[a]
Q1 of UCHI3L1	0.9	1.5	1.7	13.8
Q2 of UCHI3L1	2.0	4.7	4.5	40.0
Q3 of UCHI3L1	4.8	11.0	24.0	1413.3

[a]Based on the KDIGO criteria for AKI
Concentration of UCHI3L1 in ng/ml
Abbreviations: AKI acute kidney injury, KDIGO Kidney Disease | Improving Global Outcomes, SCr serum creatinine, UCHI3L1 urinary chitinase 3-like protein 1, UO urine output

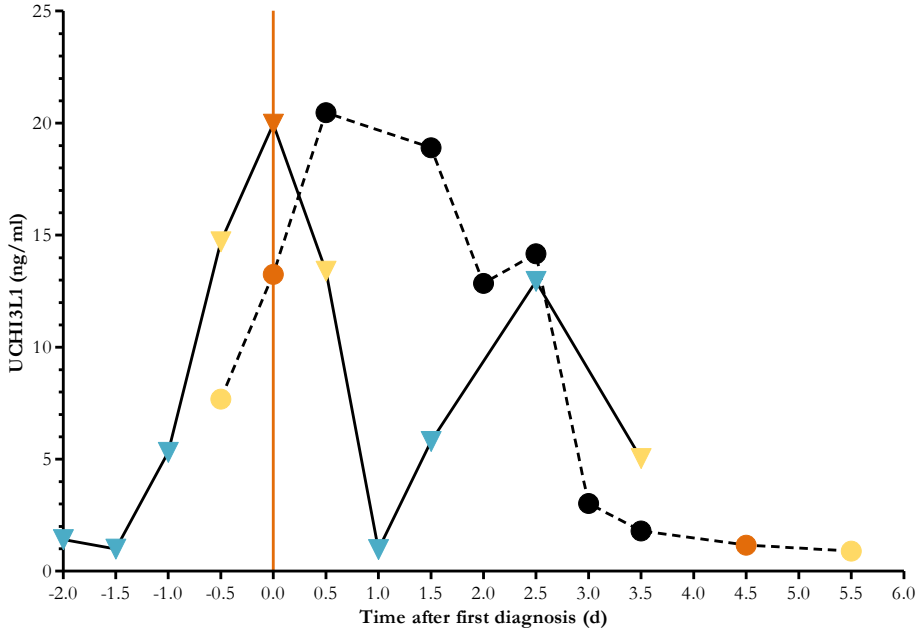


Figure 5 | Individual concentration-time-course of UCHI3L1 with the KDIGO stage-time-course for two AKI patients
The concentration of UCHI3L1 at different time points is represented by bullets in patient A (dashed line) and by triangles in patient B (full line). The colour of both symbols reflects the $AKI_{SCr/UO}$ stage of these two patients at each time point: green represents no AKI, yellow represents AKI stage 1, orange represents AKI stage 2, and black represents AKI stage 3. For both patients, the vertical line at 0.0 d represents the time of first diagnosis of $AKI_{SCr/UO}$ stage ≥ 2. Patient A had a low UCHI3L1 at 3.5 d after first diagnosis of $AKI_{SCr/UO}$ stage ≥ 2, while KDIGO still indicated $AKI_{SCr/UO}$ stage 3 at that time. Patient B had a second UCHI3L1 peak at 2.5 d after first diagnosis of $AKI_{SCr/UO}$ stage ≥ 2, while KDIGO still indicated no $AKI_{SCr/UO}$ at that time.
Abbreviations: AKI acute kidney injury, KDIGO Kidney Disease | Improving Global Outcomes, SCr serum creatinine, UCHI3L1 urinary chitinase 3-like protein 1, UO urine output

V. Discussion

We found that UCHI3L1 was a good biomarker for early detection of AKI stage ≥ 2 in adult critically ill patients admitted to an ICU, with a performance similar to that of UNGAL.

These findings may have important clinical and socioeconomic impact. Increasing severity of AKI is associated with increased risk of worse patient and kidney outcomes [35, 36, 38, 125-130]. Importantly, observational and also intervention studies showed that early AKI management can counteract AKI deterioration, and is associated with lower mortality and less RRT dependence at discharge [24, 25, 147-152]. Consequently, even earlier identification of AKI using a biomarker may have a much stronger effect on these outcomes.

Both UCHI3L1 and UNGAL better predicted AKI stage ≥ 2 that was defined based on SCr alone versus based on SCr or UO. These two urinary proteins are biomarkers of renal stress or damage [89, 110], while SCr and UO are GFR surrogates. However, UO is much more sensitive to decline in GFR, and therefore is probably associated with less renal stress or damage than SCr, which is supported by studies reporting that UO-based AKI classes are associated with a lower ICU/hospital mortality than SCr-based ones [38, 153, 154]. This may explain the better AUC-ROCs when considering SCr alone for diagnosis. The findings by Macedo et al. [155], who reported similar ICU mortality for exclusively UO+ AKI patients (8.8 %) and (non)oliguric SCr+ AKI patients (10.4 %), appear contradictory to previous findings [38, 153, 154]. However, severity of AKI was greater in exclusively UO+ patients: > 60 % of these patients were stage 2, while > 70 % of the (non)oliguric SCr+ patients were stage 1 [155]. We also observed a partial overlap in UCHI3L1 between $AKI_{SCr/UO}$ stage 1 and stage 2 samples, indicating heterogeneity of AKI severity within KDIGO classes, which can be partly explained by the different impact of meeting the defined criteria for either UO alone, or SCr alone, or both SCr and UO [38]. This could also clarify the decreased performance of UCHI3L1 and UNGAL at enrolment for prediction of $AKI_{SCr/UO}$ stage ≥ 2 within the next 24 h. The majority of the extra $AKI_{SCr/UO}$ stage ≥ 2 patients in the 24 h observation period fulfilled UO criteria only. These patients, therefore,

probably had less renal stress or damage, and consequently a low biomarker signal. Another explanation could be that the hit leading to AKI is following the biomarker measurement. This may more likely occur when the observation period is longer [136].

The observation that the AUC-ROC for the individual KDIGO parameters, i.e. SCr and UO, were similar to those of UCHI3L1 for detection of AKI stage ≥ 2, warrants discussion. First, when comparing the AUC-ROC for UO and UCHI3L1, we must take into account that although the measurement of UO started at enrolment, it was only completed 6 h later than the time at which UCHI3L1 was measured. Second, renal stress or damage may not always be reflected by decline in GFR; vice versa, a decline in GFR may not always reflect renal stress or damage. This may lead to underestimation of the diagnostic performance of UCHI3L1 in our study.

We found a trend for increased UCHI3L1 concentrations in the 24 h preceding AKI, and for decreased concentrations afterwards. However, it should be emphasized that after meeting $AKI_{SCr/UO}$ stage ≥ 2, the individual courses of $AKI_{SCr/UO}$ differed widely between patients: some patients remained in the same severity stage, some deteriorated and others ameliorated *(data not shown)*. The number of patients observed in this pilot study also precludes firm conclusions.

This is the first translational study demonstrating that UCHI3L1 predicts the occurrence of AKI stage ≥ 2 in adult critically ill patients [84]. Schmidt et al. independently showed that UCHI3L1 predicts the occurrence of delayed graft function in adult patients who receive deceased-donor kidney transplants [110]. In their preclinical study, these authors reported that the transcription of the CHI3L1 gene is significantly upregulated in the mouse kidney after IRI with increased excretion of its protein in urine. These mRNA and protein levels correlated with the degree of kidney injury and were at earliest measured on the first day after IRI, when SCr values had already peaked. Recently, the same group also studied a cohort of hospitalized patients who had AKI, and found that UCHI3L1 was associated with the composite outcome of AKI progression and in-hospital death [156].

We must speculate on the source of CHI3L1. Upon renal stress or damage, this protein is secreted by macrophages within the kidney [110], while NGAL is secreted by specific cells of the distal nephron [89]. Another source for the urine component of NGAL is the circulating plasma pool [157]. We speculate that the same is true for CHI3L1 as this protein has an apparent molecular weight of ±39-40 kDa [102, 103], and as within the group of patients with no AKI (in the 7-d prediction window) a concomitant high level of SCHI3L1 was observed more in those with an increased than with a normal UCHI3L1 at enrolment *(Supplemental Table S8)*. Additionally, we speculate that CHI3L1 binds to the megalin receptor for tubular reabsorption. This implies that NGAL and CHI3L1 can each indirectly affect the urinary concentration of the other, as they are then competitors [157].

Similar to NGAL, CHI3L1 is also stored in the secondary granules of circulating neutrophils [158-160]. This could implicate that in the urine of patients with a urinary tract infection **(UTI)**, CHI3L1 is increased too [161]. Although data on UCHI3L1 in UTI patients are missing, proteome profiling of human neutrophils suggests that this issue is less relevant for UCHI3L1 [159], which agrees with the reported 12 pg NGAL and 0.16 pg CHI3L1 per leukocyte [161, 162].

Surprisingly, only in 2013 He et al. investigated the possibility that the CLP CHI3L1 mediates its biological effects through receptor binding, and identified IL-13Rα2 as its binding partner [109]. These biological effects include inhibition of apoptosis in renal epithelial cells [109, 110], and inhibition of pyroptosis and IL-1β production in macrophages [109, 111]. These innate immune cells play an important role in both kidney injury and repair [163].

Our study has important limitations. First, this is a single-center study conducted in surgical and medical ICUs. Although the baseline characteristics of patients, the observed outcomes, and the NGAL cutoff based on the Youden index suggest that the patients included are representative of ICUs in developed countries, these data remain to be confirmed in other centers and in different types of ICU. Second, only a limited number of patients reached the primary endpoint, which can be partly explained by selection bias, i.e. not asking (legally authorized representatives of) the

most critically ill patients for consent. Yet, this is a typical and hence, rather unavoidable feature of prospective studies like this [136]. The restricted period for observation of AKI stage ≥ 2 certainly contributes to the low event rate as well. Therefore, we included all 21 patients (12 %) who developed $AKI_{SCr/UO}$ stage ≥ 2 within 7 d after enrolment in the mixed model analysis. Third, following the KDIGO guidelines [11], reference SCr was defined as the lowest SCr value within the last 3 mo prior to enrolment. This method is prone to bias, as blood draws for SCr measurement tend to be performed more often when patients are in hospital or sick, thereby not reflecting true baseline kidney function. Fourth, we did not measure urinary [TIMP-2]•[IGFBP7], a two-biomarker panel found to be superior to UNGAL [136, 139, 140], because it was not available at the start of our study.

VI. Conclusions

In summary, we demonstrated that UCHI3L1 measured in critically ill patients admitted to an ICU, predicted the occurrence of AKI stage ≥ 2 within a 12-h or 24-h observation period. The results of this pilot study need confirmation in different settings and in larger patient cohorts.

VII. Supplemental Material to Methods

SAMPLE COLLECTION AND HANDLING

Blood (serum)

Approximately 6 ml of blood was obtained via an indwelling arterial line at each study-specific sampling moment. Blood was collected in clot activator collection tubes (Venosafe 6 ml, ref. VF-106SAS, Terumo Europe, Leuven, BE) for serum. After clotting at 4°C (in weekends: storage up to ±40 h *(Supplemental Table S3A)* at 4°C based on our stability results *(Additional Table A3)*), serum samples were centrifuged (Heraeus Labofuge 400 R, swinging bucket rotor with round bucket, Thermo Fisher Scientific, Waltham, MA) at 4°C and 1992 x g for 15 min. The supernatant was divided into 4 aliquots: 1 for CHI3L1, 1 for Cr, and 2 as backup. These

eppendorf tubes containing the supernatant were immediately stored at -80°C. No preservatives were added. Samples were thawed at room temperature immediately prior to analysis and vortexed before pipetting.

Urine

Approximately 5-10 ml of urine was obtained via an indwelling bladder catheter at each study-specific sampling moment. Urine was collected directly from the catheter (never from the collection bag) via the needle-free port-system in a standard (non-coated) transport container that can also be used as centrifuge tube for sediment recovery (Urine Monovette 10 ml, ref. 10.252, Sarstedt, Nümbrecht, DE). Urine was immediately (in weekends: storage up to ±40 h *(Supplemental Table S3A)* at 4°C based on our stability results *(Additional Table A3)*) centrifuged (Heraeus Labofuge 400 R, swinging bucket rotor with round bucket, Thermo Fisher Scientific, Waltham, MA) at 4°C and 1029 x g for 10 min. The supernatant was divided into 5 aliquots: 1 for CHI3L1, 1 for NGAL, 1 for Cr, and 2 as backup. These eppendorf tubes containing the supernatant were immediately stored at -80°C. No preservatives were added. Samples were thawed at room temperature immediately prior to analysis and vortexed before pipetting. Eppendorf tubes that still contained visible sediment were very shortly (< 15 seconds) centrifuged (Heraeus Biofuge Fresco, Thermo Fisher Scientific, Waltham, MA) at 4°C and 7697 x g before pipetting.

BIOMARKER MEASUREMENTS

Creatinine

Cr concentrations were measured in the 24-h laboratory of Ghent University Hospital with a kinetic rate-blanked Jaffé assay (commercial reagents, Roche Diagnostics, Basel, CH) on a Cobas c502.

All samples were analysed within 6 mo after collection (median: 3 mo; interquartile range **(IQR)**: 2-4 mo) complying with the reported stability: when stored without preservatives at -22°C respectively -25°C, Cr was stable for 15 y in urine [164], and for 25 y in serum [165].

Chitinase 3-like protein 1

The concentration of CHI3L1 was determined in-house by *De Loor J*, *Demeyere K*, and *Van Nuffel K* with an ELISA (Human Chitinase 3-like 1 Quantikine ELISA Kit, ref. DC3L10, R&D Systems, Minneapolis, MN). The standard procedure that was followed when measuring CHI3L1 by ELISA is as follows. All samples and reagents were brought to room temperature. Samples and standards requiring dilution were accordingly prepared using calibrator diluents (1/200 or 1/500 dilution for serum; 1/5 or 1/10 dilution for urine) *(Supplemental Table S3B)*. To each well pre-coated with a rat monoclonal antibody against recombinant human CHI3L1 we added 100 µl of assay diluents followed by 50 µl of the appropriate sample or standard. This mixture was allowed to react for 2 h at room temperature. Each well was then aspirated and washed 4 times before adding 200 µl of a horseradish peroxidase-conjugated goat polyclonal antibody against recombinant human CHI3L1. After another incubation time of 2 h at room temperature, each well was again aspirated and washed 4 times. We then added 200 µl of substrate solution per well, which consisted of 100 µl of hydrogen peroxide and 100 µl of tetramethylbenzidine. This mixture was incubated in the dark for 30 min. Finally, 50 µl of stop solution was added per well after which the optical density **(OD)** of each well was measured with a microplate reader (Multiskan MS microplate reader, Thermo Fisher Scientific, Waltham, MA) set at 450 nm. The correction wavelength was set at 550 nm. The serum and urinary CHI3L1 concentrations were calculated with a microplate analysis program (DeltaSoft JV, Biomettalics, Princeton Junction, NJ). The 4-parameter logistic **(4PL)** model was chosen for curve fitting, as described by the manufacturer. All samples were analysed within 13 mo after collection (median: 7 mo; IQR: 3-10 mo). When stored without preservatives *(personal communication with Johansen JS)* at -80°C, SCHI3L1 is stable

for 8 y [166]. Additionally, we showed that UCHI3L1 may even be measured after a second thawing step within at least 30 mo after the first freezing *(Additional Table A4)*.

Neutrophil gelatinase-associated lipocalin

The concentration of UNGAL was measured in the clinical chemistry laboratory of Ghent University Hospital with a PETIA (NGAL Test, ref. ST001-3CA, BioPorto, Hellerup, DK) on a Modular P. The standard procedure that was followed when measuring UNGAL by PETIA is as follows. All samples and reagents were brought to room temperature. After calibrating and running the controls 150 µl of sample was provided in a specific sample cup, as described by the manufacturer. To read over the measuring principle of this PETIA, we refer to other literature [167].

All NGAL analyses were performed in batch in November 2014. Storage at -80°C for 2 y without preservatives has been shown not to affect UNGAL [168].

Urine output calculation

For UO calculations we accepted a margin of 10 % under the 1-h block. Therefore, all urine volume measurements over a period less than 0.9 h (54 min) were first counted up with the following measurement. Then, we redistributed all blocks ≥ 1.8 h and < 2.7 h into 2 blocks, and all blocks ≥ 2.7 h and ≤ 3 h into 3 blocks, generating blocks of ≥ 0.9 h and < 1.8 h. Blocks > 3 h were considered as unreliable for redistribution and UO calculation.

Statistical analysis

The primary analysis was based on comparison of the AUC-ROCs of UCHI3L1 with those of UNGAL for predicting the defined endpoints, which was performed in MedCalc 15.2.1 (MedCalc Software, Oostende, BE). The method by DeLong et al. was selected for calculation of the standard error of both the AUC-ROC and the difference between 2 AUC-ROCs [169]. For the AUC-ROC, a binomial exact 95 % CI was calculated. This program also lists the Youden index [170], defined as the maximum of [sensitivity plus specificity minus 1], with its associated

criterion for each AUC-ROC. We also calculated Spearman's coefficients of rank correlation with this program.

In SPSS 22 (IBM, Armonk, NY) we performed:

(a) Mixed model analysis with log10(UCHI3L1) as outcome variable, diagnosis of the 1st episode of AKI stage ≥ 2 based on the KDIGO criteria ($AKI_{SCr/UO}$) within 24 h after sampling as predictor variable, and patient as random factor.

(b) Unpaired comparison of a variable between 2 independent samples. Categorical variables were analysed with Fisher's exact or the chi-square test, and continuous variables with the nonparametric Mann-Whitney U test. The SPSS 'Descriptives' menu uses Method 6 from the article by Hyndman and Fan for calculation of the IQR [171]. Additionally, we calculated the 95 % CI for a proportion using the Wilson procedure without a correction for continuity [145, 146].

(c) Paired comparison of a continuous variable between 2 related samples *(Additional Tables A3 and A4)* using the Wilcoxon matched-pair signed-rank test.

(d) Paired comparison of a continuous variable between > 2 related samples *(Additional Table A3)* using the related- samples Friedman's two-way analysis of variance by ranks.

Box and whisker plots were generated in GraphPad Prism 5 (GraphPad Software, San Diego, CA), which also uses Method 6 for calculation of the 1st and 3rd quartile [171]. The method by Tukey was selected for drawing of the whiskers [172].

For all analyses, 2-sided P values < 0.05 were considered significant.

We will now describe how the urinary biomarkers were introduced into the statistical models.

Review and adjustment of CHI3L1 concentrations before input in statistical programs

Remark: CHI3L1 concentrations are expressed in pg/ml in these raw data.

Step 1: Evaluation of the standard curve of the ELISA

Only when the coefficient of determination (R^2) is greater than or equal to (≥) 0.995, there is a good fit of the 7 standard points in the 4PL model. Additionally, we evaluated whether the

assured dynamic range of the standard curve was in fact as dynamic as guaranteed *(Supplemental Table S3C)*.

Step 2: Re-analysis of samples with a concentration (not adjusted for dilution) outside the validated dynamic range of the curve

The details of this standard procedure are outlined in *Supplemental Table S3D*.

Step 3: Adjustment of CHI3L1 concentrations of optimally diluted samples with $OD_{sample} < OD_{62.5}$

Supplemental Table S3E in detail outlines the standard procedure that was followed in this case. Note that, based on the acceptable recovery limits (70-130 %), the back-calculated Concentration$(Conc)_{62.5}$ ranges from 43.8-81.3 pg/ml.

Adjustment of UNGAL concentrations before input in statistical programs

In the 'performance data and application note for Roche Modular P' can be read that the limit of quantification **(LOQ)** of the NGAL Test was determined to be 25 ng/ml on this analyser model. As the limit of detection **(LOD)** was not tested on this model we were advised *(personal communication with BioPorto)* to use the LOQ that was estimated on the Roche Hitachi 917, which was 12 ng/ml. The measured UNGAL concentrations were adjusted based on these reported limits *(Supplemental Table S3F)*.

SUPPLEMENTAL TABLES TO METHODS

Table S1 | STrengthening the Reporting of OBservational studies in Epidemiology statement – checklist of items that should be included in the reports of cohort studies [143]

	Item No.	STROBE recommendation	Fulfilled
Title and abstract			
	1	(a) Indicate the study's design with a commonly used term in the title or the abstract	Yes
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found	Yes
Introduction			
Background / rationale	2	Explain the scientific background and rationale for the investigation being reported	Yes
Objectives	3	State specific objectives, including any pre-specified hypotheses	Yes
Methods			
Study design	4	Present key elements of study design early in the paper	Yes
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	Yes
Participants	6	(a) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	Yes
		(b) For matched studies, give matching criteria and number of exposed and unexposed	NA
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	Yes
Data sources / measurement	8[a]	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	Yes
Bias	9	Describe any efforts to address potential sources of bias	Yes
Study size	10	Explain how the study size was arrived at	Yes
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	Yes
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding	Yes
		(b) Describe any methods used to examine subgroups and interactions	Yes
		(c) Explain how missing data were addressed	NA
		(d) If applicable, explain how loss to follow-up was addressed	NA
		(e) Describe any sensitivity analyses	NA
Results			
Participants	13[a]	(a) Report numbers of individuals at each stage of study – e.g., numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	Yes
		(b) Give reasons for non-participation at each stage	Yes
		(c) Consider use of a flow diagram	Yes
Descriptive data	14[a]	(a) Give characteristics of study participants (e.g., demographic, clinical, social) and information on exposures and potential confounders	Yes
		(b) Indicate number of participants with missing data for each variable of interest	NA
		(c) Summarize follow-up time (e.g., average and total amount)	Yes
Outcome data	15[a]	Report numbers of outcome events or summary measures over time	Yes
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (e.g., 95% confidence interval). Make clear which confounders were adjusted for and why they were included	Yes
		(b) Report category boundaries when continuous variables were categorized	NA
		(c) If relevant, consider translating estimates of relative risk into absolute	NA

	Item No.	STROBE recommendation	Fulfilled
		risk for a meaningful time period	
Other analyses	17	Report other analyses done - e.g., analyses of subgroups and interactions, and sensitivity analyses	Yes
Discussion			
Key results	18	Summarize key results with reference to study objectives	Yes
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	Yes
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	Yes
Generalizability	21	Discuss the generalizability (external validity) of the study results	Yes
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	Yes

[a]Give information separately for exposed and unexposed groups
Abbreviations: NA not applicable, No. number, STROBE STrengthening the Reporting of OBservational studies in Epidemiology

Table S2 | Kidney Disease | Improving Global Outcomes definition and classification of acute kidney injury [11]

KDIGO AKI definition	SCr increase to ≥ 1.5 times baseline, which is known or presumed to have occurred within the prior 7 d SCr increase by ≥ 0.3 mg/dl within 48 h UO < 0.5 ml/kg/h for ≥ 6 consecutive h

KDIGO AKI stage[a]	SCr	UO
1	Increase to ≥ 1.5 times baseline Increase by ≥ 0.3 mg/dl	< 0.5 ml/kg/h for ≥ 6 consecutive h
2	Increase to ≥ 2 times baseline	< 0.5 ml/kg/h for ≥ 12 consecutive h
3	Increase to ≥ 3 times baseline Increase to ≥ 4 mg/dl Initiation of RRT	< 0.3 ml/kg/h for ≥ 24 consecutive h Anuria for ≥ 12 consecutive h

[a]For staging purposes, patients should be staged according to the criterion or criteria that give(s) them the highest stage
Abbreviations: AKI acute kidney injury, KDIGO Kidney Disease | Improving Global Outcomes, RRT renal replacement therapy, SCr serum creatinine, UO urine output

Table S3A | Two representative work schemes for sample centrifugation in weekends

Work scheme 1 **Sat, 10 am**		**Mon, 8 am**	
Sample from	Time stored at 4°C (h) before centrifugation	Sample from	Time stored at 4°C (h) before centrifugation
Fri, 6 pm	16	Sat, 6 pm	38
Sat, 6 am	4	Sun, 6 am	26
		Sun, 6 pm	14
		Mon, 6 am	2
Work scheme 2 **Sun, 10 am**		**Mon, 8 am**	
Sample from	Time stored at 4°C (h) before centrifugation	Sample from	Time stored at 4°C (h) before centrifugation
Fri, 6 pm	40	Sun, 6 pm	14
Sat, 6 am	28	Mon, 6 am	2
Sat, 6 pm	16		
Sun, 6 am	4		

Based on our stability results *(Additional Table A3)*, the responsible study coordinator had to come once a weekend: on Sat or Sun.

Table S3B | Dilution of serum and urine samples for initial measurement of CHI3L1 by enzyme-linked immunosorbent assay

Serum CRP (mg/l)	Estimated dilution for serum sample	SCr (mg/dl)	Estimated dilution for urine sample
< 10	1/200	No increase to ≥ 1.5 times baseline, which is known or presumed to have occurred within the prior 7 d and no increase by ≥ 0.3 mg/dl within 48 h	1/5
≥ 10	1/500	Increase to ≥ 1.5 times baseline, which is known or presumed to have occurred within the prior 7 d or increase by ≥ 0.3 mg/dl within 48 h	1/10

The dilution of a serum sample for the initial measurement of CHI3L1 by ELISA was chosen based on a patient's serum CRP, while the dilution of a urine sample for the initial measurement was chosen based on a patient's SCr.
Abbreviations: CHI3L1 chitinase 3-like protein 1, CRP C-reactive protein, ELISA enzyme-linked immunosorbent assay, SCr serum creatinine

Table S3C | Evaluation of the assured dynamic range of the CHI3L1 enzyme-linked immunosorbent assay standard curve

Expected standard concentration (pg/ml)	Back-calculated standard concentration (pg/ml)	Recovery (%)	Acceptable recovery (%)
4000	X	(X/4000)*100	70-130
2000	X	(X/2000)*100	70-130
1000	X	(X/1000)*100	70-130
500	X	(X/500)*100	70-130
250	X	(X/250)*100	70-130
125	X	(X/125)*100	70-130
62.5	X	(X/62.5)*100	70-130

If a standard point has an unacceptable recovery while the adjacent standard points have acceptable recoveries, this point is removed from the standard curve. The effect on the novel standard curve is then reviewed.
The symbol * represents the multiplication sign, while the symbol / represents the division sign.
Abbreviations: CHI3L1 chitinase 3-like protein 1

Table S3D | Re-analysis of samples with a concentration (not adjusted for dilution) outside the validated dynamic range of the CHI3L1 enzyme-linked immunosorbent assay standard curve

Situation 1 | narrowed validated dynamic range		
OD_{sample} > $OD_{validated\ highest}$	OD_{sample} > OD_{4000}	Re-analyse and dilute more
	OD_{sample} < OD_{4000}	Re-analyse and dilute equally
OD_{sample} < $OD_{validated\ lowest}$	OD_{sample} > $OD_{62.5}$	Re-analyse and dilute equally
	OD_{sample} < $OD_{62.5}$	Re-analyse and dilute less
Situation 2 | validated dynamic range of [62.5-4000]		
OD_{sample} > OD_{4000}	Option 1	Re-analyse and dilute more
	Option 2	Do not re-analyse and report as [back-calculated $Conc_{4000}$] multiplied with [dilution factor of sample][a] Implication: most likely underestimation
OD_{sample} < $OD_{62.5}$	Option 1	If less dilution is possible: re-analyse and dilute less
	Option 2	If less dilution is possible: do not re-analyse and report as missing value[a]
	Option 3	If less dilution is not possible (sample already optimal diluted): see *Supplemental Table S3E*

[a]All samples collected at enrolment were re-analysed if needed, as CHI3L1 at enrolment is the primary dependent variable of the study.
Abbreviations: CHI3L1 chitinase 3-like protein 1, Conc concentration, OD optical density

Table S3E | Adjustment of CHI3L1 concentrations of optimally diluted samples with optical density$_{sample}$ < optical density$_{62.5}$

$Conc_{sample}$ < $LOD_{23.9}$	$Conc_{sample}$ < back-calculated $Conc_{62.5}$	0-23.8 < 23.9 < 43.8-81.3	$Conc_{sample}$ < $LOD_{23.9}$ < back-calculated $Conc_{62.5}$	Median between 0 and 23.9
$Conc_{sample}$ = $LOD_{23.9}$	$Conc_{sample}$ < back-calculated $Conc_{62.5}$	23.9 = 23.9 < 43.8-81.3	$Conc_{sample}$ = $LOD_{23.9}$ < back-calculated $Conc_{62.5}$	23.9
$LOD_{23.9}$ < $Conc_{sample}$ < $LOQ_{64.6}$	$Conc_{sample}$ < back-calculated $Conc_{62.5}$	23.9 < 24-64.4 < 43.8-64.5 < 64.6	$LOD_{23.9}$ < $Conc_{sample}$ < back-calculated $Conc_{62.5}$ < $LOQ_{64.6}$	Median between 23.9 and 64.6
	$Conc_{sample}$ ≥ back-calculated $Conc_{62.5}$	23.9 < 43.8-64.5 ≤ 43.8-64.5 < 64.6	$LOD_{23.9}$ < back-calculated $Conc_{62.5}$ ≤ $Conc_{sample}$ < $LOQ_{64.6}$	Median between 23.9 and 64.6 (even if $Conc_{sample}$ falls within standard curve range)
$Conc_{sample}$ ≥ $LOQ_{64.6}$	$Conc_{sample}$ < back-calculated $Conc_{62.5}$	64.6 ≤ 64.6-81.2 < 64.7-81.3	$LOQ_{64.6}$ ≤ $Conc_{sample}$ < back-calculated $Conc_{62.5}$	Median between 23.9 and 64.6 (even if $Conc_{sample}$ equals or exceeds $LOQ_{64.6}$)
	$Conc_{sample}$ ≥ back-calculated $Conc_{62.5}$	64.6 ≤ 64.6-81.3 ≤ 64.6-X[a]	$LOQ_{64.6}$ ≤ back-calculated $Conc_{62.5}$ ≤ $Conc_{sample}$	Measured Conc

Note that the CHI3L1 concentrations are not yet corrected for dilution.
[a]X represents a concentration > 64.6 pg/ml.
Abbreviations: CHI3L1 chitinase 3-like protein 1, Conc concentration, LOD limit of detection, LOQ limit of quantification, OD optical density

Table S3F | Adjustment of UNGAL concentrations before input in statistical programs

Measured UNGAL	Reported UNGAL (ng/ml)
< LOD_{12}	0.1
= LOD_{12}	12.0
> LOD_{12} and < LOQ_{25}	Median (LOD_{12}, LOQ_{25}) = 18.5
≥ LOQ_{25}	Measured UNGAL

Abbreviations: LOD limit of detection, LOQ limit of quantification, UNGAL urinary neutrophil gelatinase-associated lipocalin

Table S4 | Definition of suspected bacterial infection, arterial hypotension, organ dysfunction, and shock

Medication	Antibiotic drug	'Yes' or 'No'	
		When responding 'Yes'	**Suspected bacterial infection**
Arterial hypotension	Vasopressor support for at least 1 h	'Yes' or 'No'	
	MAP < 70 mmHg	'Yes' or 'No'	
Organ dysfunction	SCr > 2.0 mg/dl	'Yes' or 'No'	
	Serum bilirubin (total) > 2.0 mg/dl	'Yes' or 'No'	
	Platelet count < 100,000/µl	'Yes' or 'No'	
		When responding 'Yes' to at least one criterion	**Suspected bacterial infection leading to arterial hypotension or organ dysfunction**
Shock defined as non-responsive arterial hypotension	Vasopressor support for at least 1 h	'Yes' or 'No'	
	MAP < 65 mmHg	'Yes' or 'No'	
		When responding 'Yes' to both criteria	**Suspected bacterial infection leading to shock**

Abbreviations: MAP mean arterial pressure, SCr serum creatinine

VIII. Supplemental Material to Results

SUPPLEMENTAL TABLES TO RESULTS

Table S5 | Sharpened clinical phenotype analysis with areas under receiver-operating characteristics curves of the urinary biomarkers measured at enrolment

		$AKI_{SCr/UO}$ stage ≥ 2[a]			AKI_{SCr} stage ≥ 2[b]		
Biomarker measurement	Time window	AUC-ROC	95 % CI	Number of positives/total N	AUC-ROC	95 % CI	Number of positives/total N
Enrolment UCHI3L1	12 h	0.882	0.817-0.930	2/142	0.879	0.817-0.926	2/155
	24 h	0.631	0.541-0.715	4/126	0.879	0.815-0.927	2/147
Enrolment UNGAL	12 h	0.850	0.780-0.904	2/142	0.856	0.791-0.907	2/155
	24 h	0.654	0.564-0.736	4/126	0.852	0.784-0.905	2/147

Patients with AKI stage 1 at enrolment were excluded, as were patients that maximally reached AKI stage 1 within the respective time window.
[a]Based on the KDIGO criteria for AKI
[b]Based on the KDIGO SCr criteria for AKI
Abbreviations: AKI acute kidney injury, AUC-ROC area under the receiver-operating characteristics curve, CI confidence interval, KDIGO Kidney Disease | Improving Global Outcomes, SCr serum creatinine, UCHI3L1 urinary chitinase 3-like protein 1, UNGAL urinary neutrophil gelatinase-associated lipocalin, UO urine output

Table S6 | Spearman's coefficients of rank correlation for the urinary biomarkers measured at enrolment

		Spearman's coefficient of rank correlation	95 % CI	Number of patients
		0.615	0.515-0.698	181
AKI definition used to define subgroup	**Subgroup**	**Spearman's coefficient of rank correlation**	**95 % CI**	**Number of patients**
$AKI_{SCr/UO}$[a]	Stage 0 at enrolment	0.600	0.489-0.691	158
	Stage 1 at enrolment	0.532	0.153-0.774	23
	Stage 0 or 1 within 12 h after enrolment	0.601	0.497-0.688	175
	Stage 2 or 3 within 12 h after enrolment	0.543	[-0.480]-0.940	6
	Stage 0 or 1 within 24 h after enrolment	0.590	0.483-0.680	172
	Stage 2 or 3 within 24 h after enrolment	0.800	0.290-0.956	9
AKI_{SCr}[b]	Stage 0 at enrolment	0.595	0.484-0.686	160
	Stage 1 at enrolment	0.575	0.191-0.807	21
	Stage 0 or 1 within 12 h after enrolment	0.596	0.492-0.683	177
	Stage 2 or 3 within 12 h after enrolment	0.800	[-0.697]-0.996	4
	Stage 0 or 1 within 24 h after enrolment	0.589	0.483-0.678	176
	Stage 2 or 3 within 24 h after enrolment	0.900	0.086-0.993	5

Stage 0 represents no AKI.
The urinary biomarkers are UCHI3L1 and UNGAL.
[a]Based on the KDIGO criteria for AKI
[b]Based on the KDIGO SCr criteria for AKI
Abbreviations: AKI acute kidney injury, CI confidence interval, KDIGO Kidney Disease | Improving Global Outcomes, SCr serum creatinine, UCHI3L1 urinary chitinase 3-like protein 1, UNGAL urinary neutrophil gelatinase-associated lipocalin, UO urine output

Table S7 | Youden indexes with associated criterion values of the urinary biomarkers

		$AKI_{SCr/UO}$ stage ≥ 2[a]				AKI_{SCr} stage ≥ 2[b]			
Biomarker measurement	**Time window**	**J**	**Sens (%)**	**Spec (%)**	**Criterion value (ng/ml)**	**J**	**Sens (%)**	**Spec (%)**	**Criterion value (ng/ml)**
Enrolment UCHI3L1	12 h	0.651	83.3	81.7	> 7.6	0.814	100.0	81.4	> 7.6
	24 h	0.486	66.7	82.0	> 7.6	0.818	100.0	81.8	> 7.6
Enrolment UNGAL	12 h	0.467	66.7	80.0	> 139.0	0.802	100.0	80.2	> 139.0
	24 h	0.440	66.7	77.3	> 111.0	0.807	100.0	80.7	> 139.0

[a]Based on the KDIGO criteria for AKI
[b]Based on the KDIGO SCr criteria for AKI
Abbreviations: AKI acute kidney injury, J Youden index defined as the maximum of [sensitivity plus specificity minus 1], KDIGO Kidney Disease | Improving Global Outcomes, SCr serum creatinine, Sens sensitivity, Spec specificity, UCHI3L1 urinary chitinase 3-like protein 1, UNGAL urinary neutrophil gelatinase-associated lipocalin, UO urine output

Table S8 | Proportion of patients with a high systemic concentration of CHI3L1 at enrolment in patients who did not develop acute kidney injury and either presented with or without an increased urinary concentration of this biomarker at enrolment

Group	N (%)	Subgroup of a or b	N (%)	Subgroup of c, d, e or f	N (%)
(a) No $AKI_{SCr/UO}$ within 7 d after enrolment[a]	95 (100 %)	(c) UCHI3L1 > 7.6 ng/ml[b]	15 (16 %)	(g) SCHI3L1 > 2000 ng/ml	6 (40 %)
		(d) UCHI3L1 normal	80 (84 %)	(h) SCHI3L1 > 2000 ng/ml	2 (3 %)
(b) No AKI_{SCr} within 7 d after enrolment[c]	120 (100 %)	(e) UCHI3L1 > 7.6 ng/ml[d]	18 (15 %)	(i) SCHI3L1 > 2000 ng/ml	6 (33 %)
		(f) UCHI3L1 normal	102 (85 %)	(j) SCHI3L1 > 2000 ng/ml	5 (5 %)

[a]Based on the KDIGO criteria for AKI
[b]Criterion value of UCHI3L1 associated with the Youden index for predicting $AKI_{SCr/UO}$ stage ≥ 2 within 12 h after enrolment
[c]Based on the KDIGO SCr criteria for AKI
[d]Criterion value of UCHI3L1 associated with the Youden index for predicting AKI_{SCr} stage ≥ 2 within 24 h after enrolment
Abbreviations: AKI acute kidney injury, KDIGO Kidney Disease | Improving Global Outcomes, SCHI3L1 serum chitinase 3-like protein 1, SCr serum creatinine, UCHI3L1 urinary chitinase 3-like protein 1, UO urine output

IX. Additional Analyses and Results

METHODS

Subgroup analyses

We evaluated the biomarkers' diagnostic performances for predicting the primary endpoint as well as the 24-h AKI_{SCr} secondary endpoint in subgroups of patients. The selected variables used for grouping were: age, baseline estimated GFR **(eGFR)** calculated with the Chronic Kidney Disease Epidemiology Collaboration **(CKD-EPI)** formula, reason for ICU admission, patient's location prior to ICU admission, Sepsis-related Organ Failure Assessment **(SOFA)** score at d1, and presence of suspected bacterial infection, either leading to arterial hypotension or organ dysfunction, or leading to shock *(Supplemental Table S4)*, at d1.

Additionally, we used the same grouping variables in both patients who did not develop $AKI_{SCr/UO}$ within 7 d after enrolment and those who did not develop AKI_{SCr} within 7 d after

enrolment. The distribution of UCHI3L1 and UNGAL at enrolment in selected subgroups of 7-d no-$AKI_{SCr/UO}$ patients was plotted against the distribution in all 12-h no-$AKI_{SCr/UO}$ patients and in all those maximally reaching $AKI_{SCr/UO}$ stages 1, 2, or 3 within 12 h after enrolment. Likewise, the distribution of these biomarkers at enrolment in selected subgroups of 7-d no-AKI_{SCr} patients was plotted against the distribution in all 24-h no-AKI_{SCr} patients and in all those maximally reaching AKI_{SCr} stages 1, 2, or 3 within 24 h after enrolment.

Additional area under the receiver-operating characteristics curve analyses

Additional endpoints of the study were: $AKI_{SCr/UO}$ stage ≥ 1 within 12 h and 24 h after enrolment; AKI_{SCr} stage ≥ 1 within 12 h and 24 h after enrolment. As these endpoints include AKI stage 1, we additionally excluded the patients with AKI stage 1 at enrolment from the analysis cohort (n = 181). The number of patients in the resulting sub-cohorts was 158 ($AKI_{SCr/UO}$) and 160 (AKI_{SCr}).

Validation of the analytical stability of CHI3L1

Short-term stability of CHI3L1 in serum and urine before centrifugation

Høgdall et al. found no change in the CHI3L1 concentration when serum samples were left on the clot at 4°C for 24 h before centrifugation, however, after 72 h SCHI3L1 was significantly increased [173]. We further tested the stability of CHI3L1 in serum (n = 2) and also in urine (n = 4) of ICU patients when stored at 4°C for 6 h (urine), 24 h, and 48 h before centrifugation by comparing these concentrations with those of the immediately centrifuged samples and calculating the mean coefficient of variation **(CV)**.

Combined long-term and freeze-thaw stability of CHI3L1 in urine

SCHI3L1 is stable for 8 y when stored at -80°C without preservatives *(personal communication with Johansen JS)*. Høgdall et al. additionally showed that repetitive freezing and thawing of serum samples up to 8 times does not influence the concentration of SCHI3L1 [173].

For our study the most relevant stability feature concerning UCHI3L1 was whether UCHI3L1 measured in an aliquot that was thawed for the first time stayed stable after refreezing followed by thawing for the second time. Indeed, this was the protocol when UCHI3L1 fell outside the range of the standard curve at the first measurement. To evaluate this combined stability we compared those concentrations measured after the second thawing with those measured after the first thawing and calculated the mean CV. Total freezing times (i.e., time between first freezing and second thawing), specifically for this stability evaluation, ranged from 6-30 mo (n = 2 for 6 mo, 12 mo, 18 mo, 24 mo and 30 mo).

Partial in-house validation of the CHI3L1 enzyme-linked immunosorbent assay

Within-run precision or intra-assay variability

Of the 101 ELISA runs performed for CHI3L1 measurement (with ELISA kits from 6 different lots), 31 runs had replicate analyses of at least 1 sample or standard. These replicate samples were divided into 3 (serum) or 2 (urine) groups, i.e. low, intermediate (serum), and high, covering the analytical range of the standard curve (0.06-4.00 ng/ml). Note that for urine there was no intermediate group as there were no replicates available of urine samples with a UCHI3L1 concentration > 1.0 ng/ml and ≤ 2.5 ng/ml. Replicate analyses of the 0.25, 0.50 and 1.00 ng/ml standards were consistently performed as these constitute the middle 3 points of the standard curve. The intra-assay between-lot CV was calculated as the weighted mean of the mean intra-assay within-lot CVs.

Between-run precision or inter-assay variability

Upon inquiry it appeared that the inter-assay CVs reported by the manufacturer were generated using 40 different assays that were divided between 4 technicians. Each of them performed one ELISA per day. The 40 assays consisted of 2 different matched sets of reagents, just like 2 different kit lots *(Additional Table A1)*, so lot-to-lot variation was taken into account. Therefore, this part of the validation process was not repeated in-house. Three samples (type not specified) with a known CHI3L1 concentration (±0.50, ±1.00, and ±2.00 ng/ml) were analysed. The reported mean inter-assay between-lot CV was 5.3 % for the ±0.50 ng/ml sample, 5.8 % for the ±1.00 ng/ml sample, and 6.9 % for the ±2.00 ng/ml sample. The mean of these 3 CVs is 6.0 %.

Calculation of the limit of detection and limit of quantification

The minimal detectable dose or LOD was determined by adding 2.6 [174] standard deviations **(SD)** to the mean OD value of 10 replicates of the zero standard (i.e., calibrator diluents) and calculating the corresponding concentration (DeltaSoft JV, Biomettalics, Princeton Junction, NJ). Likewise, the minimal quantifiable dose or LOQ was determined by adding 10 [175] SDs to the mean OD value of 10 replicates of the zero standard and calculating the corresponding concentration.

Linearity check for urine

The linearity of the assay was assessed by the manufacturer using samples from apparently healthy volunteers. Because our study population consisted of critically ill patients we rechecked the linearity for the specimen type urine. More specifically, we wanted to investigate 'how far undiluted' we could go as urinary components linked with severe illness may possibly interfere with the CHI3L1 measurement by ELISA. Therefore, undiluted urine was not tested and designated as unsuitable. The reference for our serial dilution experiment (1/2 - 1/4 - 1/8 - 1/16) was the 1/2 diluted sample. The relationship between the measured (not adjusted for dilution)

and the expected (1/2 as reference) analyte concentration was investigated by linear regression analysis (GraphPad Prism 5, GraphPad Software, San Diego, CA).

RESULTS

Biomarkers' diagnostic performances in subgroups

Diagnostic performance at enrolment for prediction of $AKI_{SCr/UO}$ stage ≥ 2 within the next 12 h, could be calculated in 9 of the 12 subgroups *(Additional Figure A1)*. Likewise, diagnostic performance at enrolment for prediction of AKI_{SCr} stage ≥ 2 within the next 24 h, could be calculated in 8 of the 12 subgroups *(Additional Figure A2)*. As for UCHI3L1, this biomarker showed decreased diagnostic performance – defined as an AUC-ROC < the lowest border of the 95 % CI in the analysis cohort – for predicting the primary endpoint in patients either with a medical reason for ICU admission, or referred from either an emergency room, or operating room, or other hospital at ICU admission, or with a SOFA score < 12 at d1 [176]. Its performance for predicting the 24-h AKI_{SCr} secondary endpoint was decreased in patients either ≥ 65 y old, or with a SOFA score ≥ 12 at d1. As for UNGAL, this biomarker showed decreased diagnostic performance for predicting the primary endpoint in patients either ≥ 65 y old, or with a medical reason for ICU admission, or referred from the floor at ICU admission. In the latter 2 subgroups UNGAL could not predict the primary endpoint. Its performance for predicting the 24-h AKI_{SCr} secondary endpoint was decreased in patients ≥ 65 y old.

We found that patients referred from the floor at ICU admission who did not develop $AKI_{SCr/UO}$ within 7 d after enrolment, had higher urinary biomarker concentrations at enrolment than all patients who did not develop $AKI_{SCr/UO}$ within 12 h after enrolment (P = 0.002 for UCHI3L1; P = 0.001 for UNGAL) *(Additional Figure A3)*. Similarly, 7-d no-AKI_{SCr} patients who were referred from the floor at ICU admission showed higher enrolment concentrations of both urinary biomarkers than all 24-h no-AKI_{SCr} patients (P = 0.001 for both biomarkers) *(Additional Figure A4)*.

Additional diagnostic performances

The AUC-ROCs for predicting $AKI_{SCr/UO}$ stage ≥ 1 within 12 h and 24 h in patients with no $AKI_{SCr/UO}$ at enrolment were markedly decreased for both UCHI3L1 and UNGAL *(Additional Table A2)*. Likewise, the AUC-ROCs for predicting AKI_{SCr} stage ≥ 1 within 12 h and 24 h in patients with no AKI_{SCr} at enrolment were markedly decreased as well *(Additional Table A2)*. This can be explained by less renal stress or damage in patients with AKI stage 1.

Stability of CHI3L1 in serum and urine

Storage at 4°C up to 48 h before centrifugation had no effect on the CHI3L1 concentration in both serum and urine ($P \geq 0.05$), with a mean CV ranging from 3.8 to 3.9 % for serum and from 5.0 to 8.5 % for urine *(Additional Table A3)*.

Refreezing at -80°C followed by a second thawing step had no effect on the CHI3L1 concentration in urine ($P \geq 0.05$), even when the time between initial freezing and second thawing was 30 mo, with a mean CV ranging from 0.7 to 18.8 % *(Additional Table A4)*.

Partial in-house validation of the CHI3L1 enzyme-linked immunosorbent assay

For the human CHI3L1 standards 0.25, 0.50 and 1.00 ng/ml of the ELISA, the intra-assay between-lot CV was 2.9 % for the 0.25 ng/ml standard, 4.4 % for the 0.50 ng/ml standard, and 5.0 % for the 1.00 ng/ml standard *(Additional Table A5)*. Serum samples with a low or a high CHI3L1 concentration showed an excellent intra-assay between-lot CV of 2.6 % for the low and 3.2 % for the high concentrations *(Additional Table A6)*. For urine samples with a low CHI3L1 concentration an intra-assay between-lot CV of 4.1 % was calculated *(Additional Table A7)*. A mean intra-assay within-lot CV of 6.7 % was obtained for serum samples with an intermediate CHI3L1 concentration. Likewise, a mean intra-assay within-lot CV of 1.9 % was obtained for urine samples with a high CHI3L1 concentration.

The LOD was determined as 0.02 ng/ml, the LOQ as 0.06 ng/ml.

For all three serial dilution experiments, each being performed with another urine sample, the 95 % CI of the slope of the linear regression equation included 1, indicating 100 % recovery *(Additional Table A8)*. The corresponding R^2 was systematically ≥ 0.99.

CONCLUSIONS REGARDING THE VALIDATION OF THE ANALYTICAL STABILITY OF CHI3L1 AND THE PARTIAL IN-HOUSE VALIDATION OF THE CHI3L1 ENZYME-LINKED IMMUNOSORBENT ASSAY

As an important first step, we showed that serum and urine may be stored for at least 48 h at 4°C before centrifugation without affecting the CHI3L1 stability, in analogy with the reported data for UNGAL [177]. Based on these results the study personnel could flexibly plan the handling of the samples, especially in weekends *(Supplemental Table S3A)*. When subsequently stored at -80°C, UCHI3L1 may even be measured after a second thawing step within at least 30 mo after the first freezing, in analogy with the reported data for SCHI3L1 [166, 173]. This information was required for our laboratory agenda, and is also relevant for biobanking purposes. Additionally, we can guarantee that our reported CHI3L1 concentrations are accurate and reproducible.

ADDITIONAL TABLES

Table A1 | Experimental setup for evaluation of between-run precision or inter-assay variability of the CHI3L1 enzyme-linked immunosorbent assay by the manufacturer

Technician 1		Technician 2		Technician 3		Technician 4	
Reagent set 1	Reagent set 2	Reagent set 1	Reagent set 2	Reagent set 1	Reagent set 2	Reagent set 1	Reagent set 2
Day 1	Day 6	Day 1	Day 6	Day 1	Day 6	Day 1	Day 6
Day 2	Day 7	Day 2	Day 7	Day 2	Day 7	Day 2	Day 7
Day 3	Day 8	Day 3	Day 8	Day 3	Day 8	Day 3	Day 8
Day 4	Day 9	Day 4	Day 9	Day 4	Day 9	Day 4	Day 9
Day 5	Day 10	Day 5	Day 10	Day 5	Day 10	Day 5	Day 10
Within-lot CV by tech 1	Within-lot CV by tech 1	Within-lot CV by tech 2	Within-lot CV by tech 2	Within-lot CV by tech 3	Within-lot CV by tech 3	Within-lot CV by tech 4	Within-lot CV by tech 4
Between-lot CV by tech 1		Between-lot CV by tech 2		Between-lot CV by tech 3		Between-lot CV by tech 4	
Reported by manufacturer: mean of between-lot CVs							

Day X of technician 1 is not necessarily the same as day X of technicians 2-4.
Abbreviations: CHI3L1 chitinase 3-like protein 1, CV coefficient of variation

Table A2 | Areas under receiver-operating characteristics curves of the urinary biomarkers measured at enrolment for prediction of additional endpoints

		$AKI_{SCr/UO}$ stage ≥ 1[a]			AKI_{SCr} stage ≥ 1[b]		
Biomarker measurement	Time window	AUC-ROC	95 % CI	Number of positives (%)	AUC-ROC	95 % CI	Number of positives (%)
Enrolment UCHI3L1	12 h	0.614	0.534-0.691	18 (11.4)	0.647	0.568-0.721	7 (4.4)
	24 h	0.554	0.473-0.633	36 (22.8)	0.603	0.523-0.680	15 (9.4)
Enrolment UNGAL	12 h	0.553	0.472-0.632	18 (11.4)	0.503	0.423-0.583	7 (4.4)
	24 h	0.542	0.461-0.621	36 (22.8)	0.504	0.424-0.584	15 (9.4)

Patients with AKI stage 1 at enrolment were excluded.
[a]Based on the KDIGO criteria for AKI
[b]Based on the KDIGO SCr criteria for AKI
Abbreviations: AKI acute kidney injury, AUC-ROC area under the receiver-operating characteristics curve, CI confidence interval, KDIGO Kidney Disease | Improving Global Outcomes, SCr serum creatinine, UCHI3L1 urinary chitinase 3-like protein 1, UNGAL urinary neutrophil gelatinase-associated lipocalin, UO urine output

Table A3 | Short-term stability of CHI3L1 in serum and urine before centrifugation

Time between sampling and centrifugation (h)	Conc at X h[a] (ng/ml)	Conc at 0 h[b] (ng/ml)	Mean	SD	CV (%)	Mean CV (%)	P value[c]
Serum							
24	1035.3	1060.2	1047.72	17.62	1.7	3.8	0.180
	1396.5	1514.9	1455.70	83.75	5.8		
48	978.3	1060.2	1019.23	57.91	5.7	3.9	0.655
	1559.7	1514.9	1537.32	31.68	2.1		
Urine							
6	1050.3	1035.8	1043.03	10.30	1.0	6.3	0.655
	577.4	679.2	628.29	71.99	11.5		
24	3.6	3.6	3.59	0.06	1.6	8.5	0.593
	12.5	15.7	14.13	2.27	16.0		
	1096.1	1035.8	1065.93	42.68	4.0		
	571.1	679.2	625.17	76.40	12.2		
48	3.9	3.6	3.77	0.20	5.2	5.0	0.715
	14.1	15.7	14.89	1.19	8.0		
	1039.2	1035.8	1037.46	2.42	0.2		
	619.0	679.2	649.08	42.59	6.6		

[a]Concentration of sample that was stored for X h at 4°C before centrifugation
[b]Concentration of sample that was centrifuged immediately after collection
[c]The P values are the significance levels between samples that were immediately centrifuged and those stored for 6 h, 24 h or 48 h at 4°C before centrifugation
Abbreviations: CHI3L1 chitinase 3-like protein 1

Table A4 | Combined long-term and freeze-thaw stability of CHI3L1 in urine

Time between first freezing and second thawing (mo)	Conc 2[a] (ng/ml)	Conc 1[b] (ng/ml)	Mean	SD	CV (% minus 6.0 %)[c]	Mean CV (%)	P value[d]
6	2.3	3.0	2.65	0.48	12.3	18.8	0.655
	0.7	0.4	0.54	0.17	25.3		
12	4.2	3.8	4.03	0.30	1.3	0.7	0.180
	8.6	8.1	8.35	0.37	0.0[e]		
18	4.3	3.7	3.97	0.43	4.8	3.6	0.655
	9.5	10.7	10.12	0.85	2.4		
24	2.0	1.9	1.95	0.10	0.0[e]	11.9	0.655
	0.6	0.9	0.72	0.21	23.7		
30	14.0	11.9	12.94	1.46	5.3	2.6	0.180
	6.3	5.9	6.09	0.32	0.0[e]		

[a]Concentration of sample measured after first thawing of an aliquot; the samples in this stability study were initially analysed after a period ranging from 1-9 mo.
[b]Concentration of sample measured after second thawing of an aliquot
[c]Mean inter-assay CV reported by the manufacturer = 6.0 % *(Additional Table A1)*
[d]The P values are the significance levels between samples that were thawed for the first time and those thawed for the second time
[e]CV of (Conc 2, Conc 1) ≤ mean inter-assay CV of 6.0 %
Abbreviations: CHI3L1 chitinase 3-like protein 1, Conc concentration, CV coefficient of variation, SD standard deviation

Table A5 | Within-run precision or intra-assay variability of the CHI3L1 enzyme-linked immunosorbent assay for standard points

Analyte concentration (ng/ml)	Standards (n)	Repetitions (n)	Mean	SD	CV (%)	Between-lot CV with range of mean within-lot CV (%)
Standard 0.3	4	2	0.25[a]	0.00	0.9	2.9 (2.6-3.9)
			0.25[a]	0.01	3.2	
			0.23[a]	0.01	3.7	
			0.25[b]	0.01	3.9	
Standard 0.5	11	2	0.49[a]	0.02	5.1	4.4 (3.5-4.6)
			0.46[a]	0.03	6.9	
			0.52[a]	0.01	2.6	
			0.53[a]	0.02	4.2	
			0.51[b]	0.02	3.5	
			0.49[c]	0.02	3.9	
			0.46[a]	0.03	6.9	
			0.49[a]	0.01	2.6	
			0.48[d]	0.02	4.2	
			0.49[d]	0.02	4.9	
			0.49[a]	0.02	4.2	
Standard 1.0	18	2	1.01[a]	0.02	2.4	5.0 (2.4-5.8)
		2	0.95[b]	0.07	7.5	
		2	1.02[b]	0.00	0.3	
		2	0.97[b]	0.02	1.9	
		2	0.97[b]	0.03	2.7	
		2	0.94[b]	0.04	4.2	
		2	0.97[c]	0.04	3.8	
		2	0.95[c]	0.03	3.1	
		2	0.94[c]	0.03	3.5	
		2	0.98[b]	0.03	2.8	
		2	0.96[b]	0.10	10.3	
		2	0.97[b]	0.02	2.5	
		4	1.10[c]	0.10	9.2	
		2	0.95[c]	0.07	7.4	
		2	0.94[c]	0.07	7.7	
		2	0.93[b]	0.11	12.3	
		2	1.05[b]	0.05	4.7	
		2	0.98[b]	0.04	3.6	

Letters in superscript indicate different lots within each group. In the 3 different groups, letter X does not necessarily represent the same lot.
Abbreviations: CHI3L1 chitinase 3-like protein 1, CV coefficient of variation, SD standard deviation

Table A6 | Within-run precision or intra-assay variability of the CHI3L1 enzyme-linked immunosorbent assay for serum samples

Analyte concentration (ng/ml)	Serum samples (n)	Repetitions (n)	Mean	SD	CV (%)	Between-lot CV with range of mean within-lot CV (%)
Low	5	2	0.45[a]	0.02	3.9	2.6 (2.1-2.8)
			0.86[a]	0.01	1.2	
			0.73[a]	0.01	1.2	
			0.69[b]	0.01	2.1	
			0.95[a]	0.05	4.8	
Intermediate > 1.0	2	2	1.21[a]	0.02	1.8	(6.7)
		4	2.22[a]	0.26	11.6	
High > 2.5	3	2	3.01[a]	0.05	1.7	3.2 (1.7-3.9)
			3.08[b]	0.1	3.1	
			3.24[b]	0.15	4.6	

Highlighted results represent the mean, SD and CV of the OD value (out of dynamic range of the curve).
Letters in superscript indicate different lots within each group. In the 3 different groups, letter X does not necessarily represent the same lot.
Abbreviations: CHI3L1 chitinase 3-like protein 1, CV coefficient of variation, OD optical density, SD standard deviation

Table A7 | Within-run precision or intra-assay variability of the CHI3L1 enzyme-linked immunosorbent assay for urine samples

Analyte concentration (ng/ml)	Urine samples (n)	Repetitions (n)	Mean	SD	CV (%)	Between-lot CV with range of mean within-lot CV (%)
Low	9	2	0.21[a]	0.00	0.3	4.1 (1.6-9.3)
			0.07[a]	0.00	2.8	
			0.17[a]	0.00	1.7	
			0.18[a]	0.00	0.8	
			0.20[a]	0.01	3.7	
			0.20[b]	0.03	14.8	
			0.09[c]	0.00	1.6	
			0.94[a]	0.07	7.0	
			0.70[b]	0.03	3.9	
High > 2.5	2	2	2.52[a]	0.06	2.3	(1.9)
			2.74[a]	0.04	1.4	

Highlighted results represent the mean, SD and CV of the OD value (out of dynamic range of the curve).
Letters in superscript indicate different lots within each group. In the 2 different groups, letter X does not necessarily represent the same lot.
Abbreviations: CHI3L1 chitinase 3-like protein 1, CV coefficient of variation, OD optical density, SD standard deviation

Table A8 | Assessment of the enzyme-linked immunosorbent assay linearity for CHI3L1

Urine sample	Dilution	Expected concentration	Measured concentration	Slope (95 % CI)	R^2
1	1/2	0.953	0.953	0.98 (0.75-1.21)	0.99
	1/4	0.476	0.545		
	1/8	0.238	0.271		
	1/16	0.119	0.136		
2	1/2	2.248	2.248	0.94 (0.72-1.15)	0.99
	1/4	1.124	1.312		
	1/8	0.562	0.720		
	1/16	0.281	0.387		
3	1/2	2.569	2.569	0.97 (0.93-1.01)	1.00
	1/4	1.284	1.345		
	1/8	0.642	0.719		
	1/16	0.321	0.381		

Abbreviations: CHI3L1 chitinase 3-like protein 1, CI confidence interval, OD optical density, R^2 coefficient of determination

ADDITIONAL FIGURES

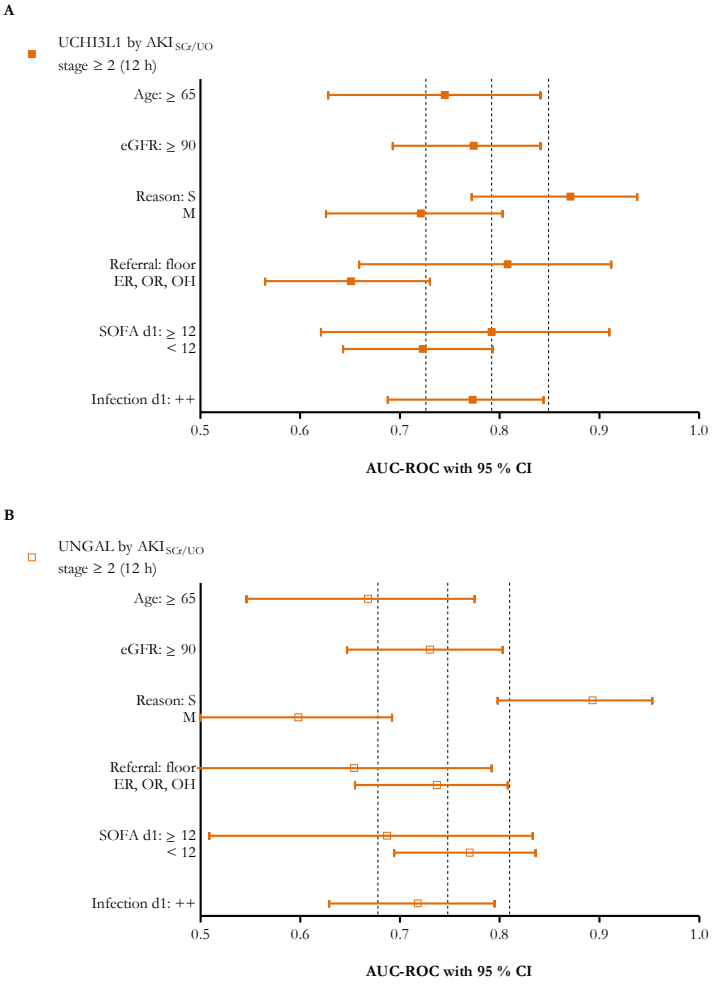


Figure A1 | Areas under receiver-operating characteristics curves of (A) UCHI3L1 and (B) UNGAL at enrolment for predicting AKI stage ≥ 2 based on the KDIGO criteria ($AKI_{SCr/UO}$) within 12 h in different subgroups of patients

The dotted vertical lines delineate the AUC-ROC with 95 % CI in the analysis cohort. The total number of patients in each of the 9 subgroups (top-down) was 71, 134, 73, 108, 43, 138, 35, 146, and 122. The definition of infection ++ is outlined in *Supplemental Table S4*.

Abbreviations: AKI acute kidney injury, AUC-ROC area under the receiver-operating characteristics curve, CI confidence interval, eGFR estimated glomerular filtration rate, ER emergency room, KDIGO Kidney Disease | Improving Global Outcomes, M medical, OH other hospital, OR operating room, S surgical, SCr serum creatinine, SOFA Sepsis-related Organ Failure Assessment, UCHI3L1 urinary chitinase 3-like protein 1, UNGAL urinary neutrophil gelatinase-associated lipocalin, UO urine output

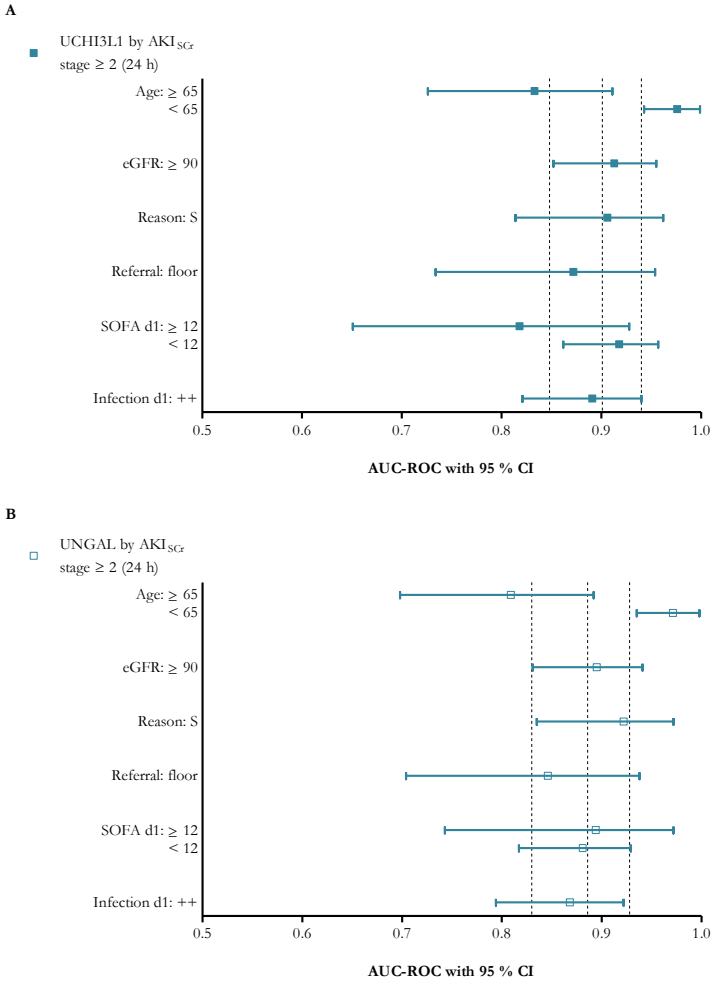


Figure A2 | Areas under receiver-operating characteristics curves of (A) UCHI3L1 and (B) UNGAL at enrolment for predicting AKI stage ≥ 2 based on the KDIGO SCr criteria (AKI_{SCr}) within 24 h in different subgroups of patients

The dotted vertical lines delineate the AUC-ROC with 95 % CI in the analysis cohort. The total number of patients in each of the 8 subgroups (top-down) was 71, 110, 134, 73, 43, 35, 146, and 122. The definition of infection ++ is outlined in *Supplemental Table S4*.

Abbreviations: AKI acute kidney injury, AUC-ROC area under the receiver-operating characteristics curve, CI confidence interval, eGFR estimated glomerular filtration rate, KDIGO Kidney Disease | Improving Global Outcomes, S surgical, SCr serum creatinine, SOFA Sepsis-related Organ Failure Assessment, UCHI3L1 urinary chitinase 3-like protein 1, UNGAL urinary neutrophil gelatinase-associated lipocalin

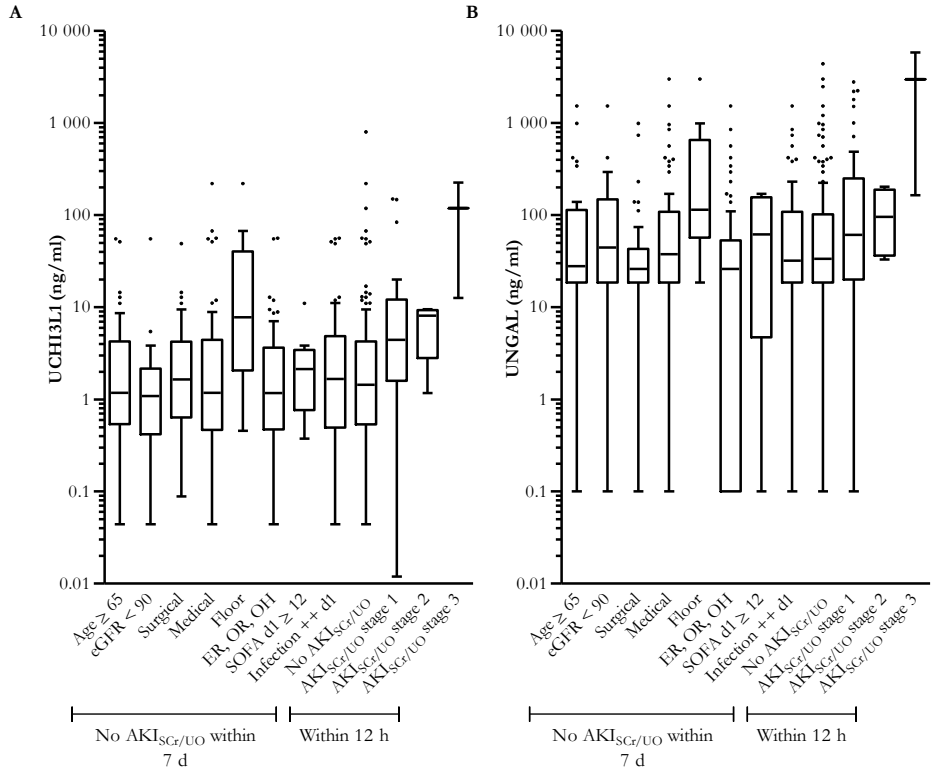


Figure A3 | Distribution of (A) UCHI3L1 and (B) UNGAL at enrolment in selected subgroups of patients who did not develop AKI based on the KDIGO criteria (no-$AKI_{SCr/UO}$) within 7 d after enrolment, compared to the distribution in all 12-h no-$AKI_{SCr/UO}$ patients, and in all those maximally reaching $AKI_{SCr/UO}$ stages 1, 2, or 3 within 12 h after enrolment

The total number of patients in each group (left-right) was 33, 16, 39, 56, 16, 79, 8, 56, 140, 35, 4, and 2. The definition of infection ++ is outlined in *Supplemental Table S4*.

Abbreviations: AKI acute kidney injury, AUC-ROC area under the receiver-operating characteristics curve, CI confidence interval, eGFR estimated glomerular filtration rate, ER emergency room, KDIGO Kidney Disease | Improving Global Outcomes, OH other hospital, OR operating room, SCr serum creatinine, SOFA Sepsis-related Organ Failure Assessment, UCHI3L1 urinary chitinase 3-like protein 1, UNGAL urinary neutrophil gelatinase-associated lipocalin, UO urine output

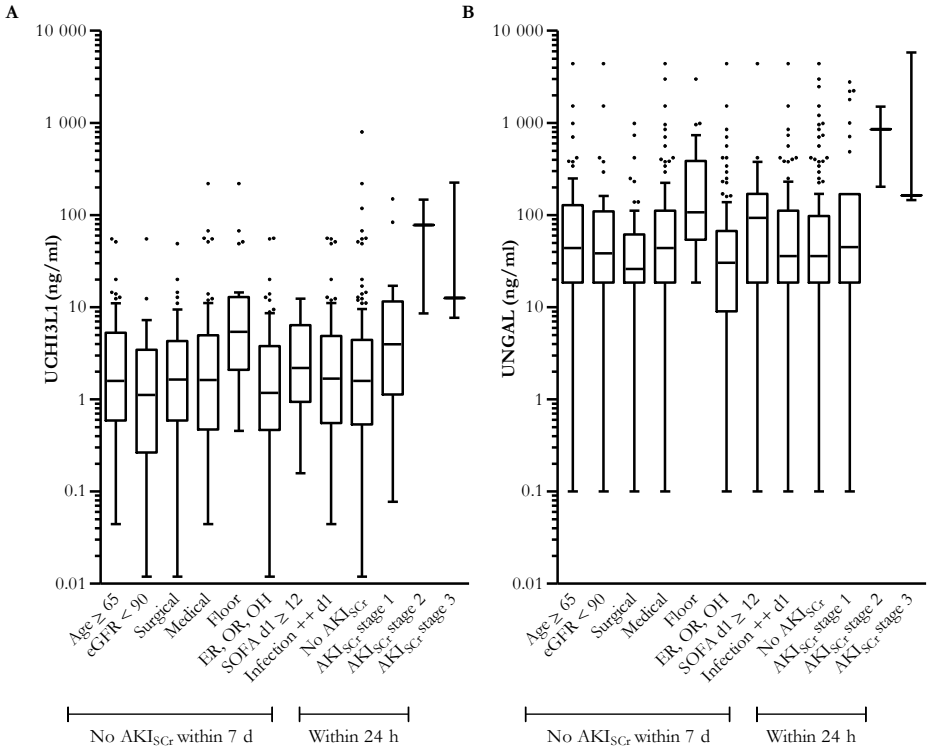


Figure A4 | Distribution of (A) UCHI3L1 and (B) UNGAL at enrolment in selected subgroups of patients who did not develop AKI based on the KDIGO SCr criteria (no-AKI_{SCr}) within 7 d after enrolment, compared to the distribution in all 24-h no-AKI_{SCr} patients, and in all those maximally reaching AKI_{SCr} stages 1, 2, or 3 within 24 h after enrolment

The total number of patients in each group (left-right) was 49, 28, 45, 75, 22, 98, 15, 76, 145, 31, 2, and 3. The definition of infection ++ is outlined in *Supplemental Table S4*.

Abbreviations: AKI acute kidney injury, AUC-ROC area under the receiver-operating characteristics curve, CI confidence interval, eGFR estimated glomerular filtration rate, ER emergency room, KDIGO Kidney Disease | Improving Global Outcomes, OH other hospital, OR operating room, SCr serum creatinine, SOFA Sepsis-related Organ Failure Assessment, UCHI3L1 urinary chitinase 3-like protein 1, UNGAL urinary neutrophil gelatinase-associated lipocalin

The following figures were added to this dissertation and are not found in the published paper.

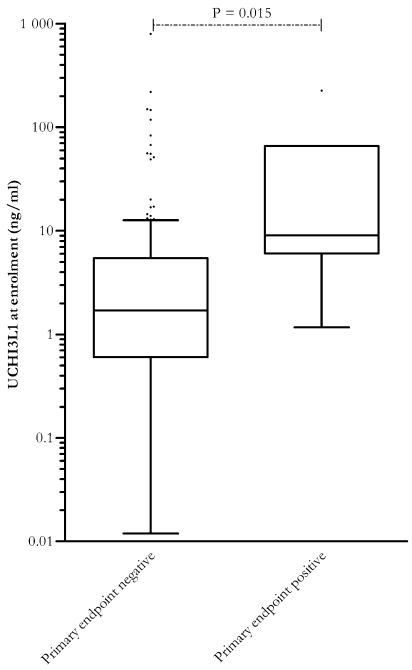


Figure A5 | Distribution of UCHI3L1 measured at enrolment in patients who either developed or did not develop AKI stage ≥ 2 based on the KDIGO criteria ($AKI_{SCr/UO}$) within 12 h after enrolment (primary endpoint)
Abbreviations: AKI acute kidney injury, KDIGO Kidney Disease | Improving Global Outcomes, SCr serum creatinine, UCHI3L1 urinary chitinase 3-like protein 1, UO urine output

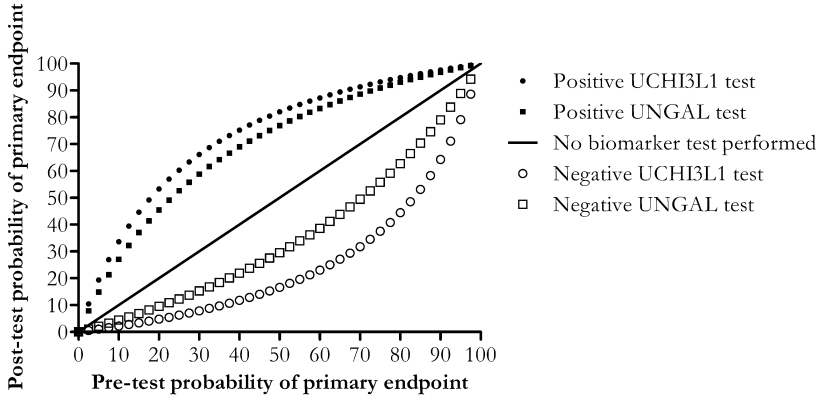


Figure A6 | Revised probability of AKI stage ≥ 2 based on the KDIGO criteria ($AKI_{SCr/UO}$) within 12 h after enrolment (primary endpoint)
This figure shows the revised (post-test) probability of the primary endpoint (y-axis) as a function of prior (pre-test) probability (x-axis) of the primary endpoint for positive and negative biomarker results of both enrolment UCHI3L1 and enrolment UNGAL, based on the likelihood ratios (data not shown) associated with the criterion values *(shown in Table S7)* of these biomarkers.

Chapter 4

Clinical Validation

of the Biomarker UCHI3L1
as Early Diagnostic or 'Predictive' Tool for Emerging AKI
in Adult ICU Patients
who Underwent Elective Cardiac Surgery

Adapted from

DOI 10.1186/s13613-017-0251-z

De Loor J, Herck I, et al., (2017) Diagnosis of cardiac surgery-associated acute kidney injury: differential roles of creatinine, chitinase 3-like protein 1 and neutrophil gelatinase-associated lipocalin: a prospective cohort study. "Annals of intensive care [electronic resource]" DOI 10.1186/s13613-017-0251-z

I. Abstract

Background: A common and serious complication of cardiac surgery prompting early detection and intervention is cardiac surgery-associated AKI **(CSA-AKI)**. UCHI3L1 was found to predict AKI associated with critical illness in adults. Our aims were therefore to evaluate whether UCHI3L1 can also be used to predict AKI associated with elective cardiac surgery in adults, and to compare this predictive ability with that of UNGAL, more frequently assessed early SCr measurements, and various two-biomarker panels.

Methods: This was a single-centre prospective cohort study at the 8-bed cardiac surgery ICU of Ghent University Hospital. AKI was diagnosed and classified according to the KDIGO definitions for the diagnosis and staging of AKI, which are based on SCr and UO. Of the 211 enrolled elective cardiac surgery patients, we included 203 patients who had no AKI pre-operatively and at time of post-operative ICU admission **(t1)** in the primary endpoint analysis (i.e., AKI stage ≥ 1 within 48 h after t1), while 210 patients without AKI stage ≥ 2 pre-operatively and at t1 were included in the secondary endpoint analysis (i.e., AKI stage ≥ 2 within 12 h after t1). Systemic and/or urine concentrations of Cr, CHI3L1 and NGAL were measured more frequently than SCr in routine early post-operative ICU practice. UO was monitored hourly in the ICU.

Results: Within 48 h after t1, 46.8 % of the patients had developed AKI (70.5 % stage 1, 20.0 % stage 2 and 9.5 % stage 3). In the early post-operative period, only SCr was a good predictor of AKI within 48 h after t1 (primary endpoint). SCHI3L1 combined with either UCHI3L1 or UNGAL was a good predictor of AKI stage ≥ 2 within 12 h after t1 (secondary endpoint). However, SCr and its absolute difference from pre-operative to early measures after surgery outperformed these combinations.

Conclusions: We found that more frequent assessment of the functional biomarker SCr in the early post-operative ICU period (first 4 h) after elective cardiac surgery in adult patients had good

to excellent predictive value for CSA-AKI, indicating that routine SCr assessment must become more frequent in order to detect AKI more early. This performance was in contrast with the inadequate predictive value of the urinary renal stress or damage biomarkers UCHI3L1 and UNGAL.

II. Background

CSA-AKI is a common and serious complication of cardiac surgery [178]. Depending on its severity and differences in both baseline characteristics and type of cardiac surgical procedure, the range of incidence of CSA-AKI is between 3.1 and 50.0 % when applying KDIGO-like criteria [179-183]. CSA-AKI treated with RRT presents post-operatively in 2.0-6.0 % of cardiac surgery patients [179, 184, 185], of which 1 out of 2 die in hospital [179]. Importantly, also when the injury is mild, CSA-AKI is independently associated with significant effects on early (i.e., hospital or 30-d) mortality [186, 187].

Risk for CSA-AKI is increased by the presence of established pre-operative (e.g., increased SCr) and peri-operative (e.g., low cardiac output) factors that increase susceptibility to AKI [188, 189]. Moreover, potentially important modifiable intra-operative susceptibilities are the type of cardiac surgical technique and, if cardiopulmonary bypass **(CPB)** (synonym: extracorporeal circulation **(ECC)**) is used, the characteristics of the perfusion technique [190]. Early CSA-AKI occurs within 7 d after the cardiac surgical procedure and is directly related to it [17]. CSA-AKI more than 1 w but within 30 d after the cardiac surgical procedure is mainly related to another exposure that presents as a complication of the cardiac surgical procedure (e.g., sepsis) [17].

Only recently the term 'acute kidney stress' **(AKS)** was proposed to describe the pre-injury phase that leads to AKI [80]. Biomarkers, or "measurable and quantifiable biological parameters" [81], that respond to AKS open new horizons in regard to the prediction and early detection of emerging AKI. These biomarkers serve as surrogate measurements, estimating renal cell perfusion, function or metabolism [80]. As regulator of the iron metabolism, NGAL measured in

blood or urine represents a way to monitor initial damage [191], which was confirmed in more than 7000 paediatric and adult patients who underwent cardiac surgery [135, 138, 192]. Recently, our group demonstrated the role of UCHI3L1 as predictor of AKI in an adult general ICU cohort, with a performance similar to that of UNGAL [193].

Higher systemic concentrations of CHI3L1 have been independently associated with the presence of coronary artery disease, seeming a quantitative indicator of disease progression as well [194]. Additionally, it was shown that in patients with type 1 and type 2 diabetes mellitus higher systemic concentrations of CHI3L1 were associated with progressing vascular damage in the kidneys, as assessed by the level of albuminuria [194]. It seems plausible that low-grade inflammation and endothelial dysfunction progressing to micro- and macrovascular complications account for higher systemic concentrations of CHI3L1. Besides a marker of chronic inflammation, systemic CHI3L1 is a marker of acute inflammation as well [96]. Cardiac surgery and the use of CPB have a critical role in inducing the systemic inflammatory response syndrome leading to CSA-AKI. Consequently, SCHI3L1 can be proposed as a potential AKI risk factor.

The objectives of this study were: ***(a)*** to evaluate whether UCHI3L1 can be used to predict occurrence of AKI in adult patients who underwent elective cardiac surgery, ***(b)*** to compare this predictive ability with that of UNGAL, a well-known biomarker of tubular damage, and that of more frequently assessed early measurements of SCr, a routine biomarker of kidney dysfunction, ***(c)*** to evaluate whether combining either two urinary renal stress or damage biomarkers, or a systemic kidney dysfunction biomarker and a urinary renal stress or damage biomarker can improve the predictive ability for CSA-AKI, ***(d)*** to evaluate whether combining SCHI3L1 with either a systemic kidney dysfunction biomarker or a urinary renal stress or damage biomarker can improve the predictive ability for CSA-AKI.

III. Methods *(see also section **VII.** "Supplemental Material")*

This research is reported according to the STROBE Statement *(Supplemental Table S1)* [143].

DEFINING AKI

AKI was diagnosed and classified according to the KDIGO definitions for the diagnosis and staging of AKI, which are based on SCr and UO *(Supplemental Figure S1)* [11].

STUDY PATIENTS

In this single-centre cohort study, we prospectively enrolled patients who were admitted to the 8-bed cardiac surgery ICU of Ghent University Hospital from May 2012 till February 2014. The inclusion and exclusion criteria of the study are incorporated in the flow diagram in **Figure 1**.

PRIMARY ENDPOINT

The primary endpoint of the current study was the development of AKI stage ≥ 1 within 48 h after ICU admission **(t1)**. Reference SCr, representing baseline SCr, was defined as the lowest SCr value within the last 3 mo prior to enrolment (lowest of history SCr value(s) and pre-operative SCr). In our cohort 30.0 % of the patients had only the pre-operative SCr value available. In 49.3 % the lowest history SCr was lower than or equal to the pre-operative SCr, while in 20.7 % the pre-operative SCr was lower than the lowest history SCr. The details for calculation of UO are outlined by our group [21]. Note that UO was registered hourly in the ICU only.

SECONDARY ENDPOINT

The secondary endpoint was AKI stage ≥ 2 within 12 h after t1.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The Ethical Committee of Ghent University Hospital approved this study (Belgian Registration Number of the study: B670201213147). All patients or their legally authorized representatives

provided written informed consent. We respected the Declaration of Helsinki and the Good Clinical Practice Guidelines.

PROSPECTIVE SAMPLE AND DATA COLLECTION

The first collection of blood and urine was after the induction of anaesthesia and before the start of surgery (time 0 **(t0)** on the day of surgery **($d_{surgery}$)**). The rest of the specimens (n = 7) were collected post-operatively, starting at ICU admission **(t1)** and then at 2 h **(t2)**, 4 h **(t3)**, 6 h, 12 h, 24 h and 48 h after ICU admission. If the patient was discharged to the Midcare unit before 24 h or 48 h, those samples were collected there. Whenever possible, the routine collection times were followed (i.e., at 4 pm on the first post-operative day **($d_{1post\text{-}op}$)** and at 6 am on the second post-operative day **($d_{2post\text{-}op}$)**). The sample collection times for a fictional patient who underwent surgery in the morning are outlined on the timeline in *Supplemental Figure S2A*, while those for a fictional patient who underwent surgery in the afternoon are outlined on the timeline in *Supplemental Figure S2B*.

These paired blood and urine samples were collected by standard methods and centrifuged by standard protocols, as described in *Chapter 3* [193]. Serum and urine supernatants were stored at -80°C and thawed at room temperature immediately prior to analysis. Clinical data needed to complete the individual clinical research files *(Supplemental Table S2)* were extracted from the hospital records by study coordinators. Samples were anonymized as were clinical data. All technicians were blinded to clinical data.

BIOMARKER ANALYSIS AND SINGLE-BIOMARKER DIAGNOSTIC TEST POSSIBILITIES

The CHI3L1 analysis was performed in-house. We measured the concentration of CHI3L1 by a sandwich ELISA technique (DC3L10, R&D Systems, Minneapolis, MN, USA). Analyses performed externally were Cr and UNGAL. The Cobas c502 measured the concentration of Cr by a kinetic rate-blanked Jaffé assay (commercial reagents, Roche Diagnostics, Basel, Switzerland), whereas the Modular P measured the concentration of UNGAL by a PETIA

technique (ST001-3CA, BioPorto, Hellerup, Denmark). All details were described in *Chapter 3* [193], except for the standard sample dilution scheme used in the CHI3L1 ELISA, which is presented in *Supplemental Table S3*. For blood samples that were collected at routine collection times, a SCr concentration was already available in the hospital records. Based on the temporal relationship of the predictive value of UNGAL for CSA-AKI [135], we measured this biomarker at t1 and t3 only.

Besides UCHI3L1 and UNGAL, we also evaluated UCHI3L1 and UNGAL corrected for urine dilution by using the ratio to UCr as diagnostic test. Besides SCr, we also evaluated ΔSCr_{tx-t0} as diagnostic test, representing the absolute change in SCr between SCr_{tx} and SCr_{t0}. The most recent SCr value recorded prior to surgery was considered as SCr_{t0}.

DEFINING ACUTE TUBULAR DAMAGE AND SUBCLINICAL AKI

Following the recommendations of de Geus et al., acute tubular damage was defined as a CSA-NGAL score of 2 or greater, i.e. either as $UNGAL_{t1}$ or $UNGAL_{t3} \geq 150$ ng/ml or as $\Delta UNGAL_{t3-t1} > 100$ ng/ml with $UNGAL_{t3} \geq 125$ ng/ml [195]. Subclinical AKI was defined when there was acute tubular damage (according to the 'de Geus criteria') and absence of AKI according to the KDIGO definition.

DEFINING GOOD AND EXCELLENT BIOMARKERS

An AUC-ROC of 0.750 or greater was considered to represent a good biomarker, whereas an AUC-ROC of 0.900 or greater was considered to represent an excellent biomarker [196].

STATISTICAL ANALYSIS

The principal statistical analysis was based on comparison of the AUC-ROCs of UCHI3L1 with those of UNGAL, more frequently assessed early measurements of SCr, and various two-biomarker panels for predicting both defined endpoints. It was performed in MedCalc 15.2.1 (MedCalc Software, Oostende, Belgium). The unpaired comparison of a variable between two independent samples was done in SPSS 22 (IBM, Armonk, NY, USA). Categorical variables were

analysed with Fisher's exact or the Chi-square test, and continuous variables with the nonparametric Mann-Whitney U test. Additionally, we calculated the 95 % CI for a proportion using the Wilson procedure without a correction for continuity [145, 146]. For all analyses, two-sided P values < 0.05 were considered statistically significant. All details and a description of how the biomarkers were introduced into the statistical programs are provided in *Chapter 3* [193].

IV. Results *(see also section **VII.** "Supplemental Material")*

INCIDENCE OF THE PRIMARY ENDPOINT, CHARACTERISTICS OF THE PATIENTS AND PROCEDURES, AND SHORT-TERM PATIENT OUTCOMES

The flow of patients during the study is illustrated in **Figure 1**. The initial enrolment cohort consisted of 211 patients. In the primary endpoint analysis, 8 patients who already had AKI stage ≥ 1 at t0 (n = 3) or t1 were excluded, resulting in a total number of 203 patients for analysis. Within 48 h after t1, 95 patients (46.8 %) had developed AKI: 67 (70.5 %) were classified as stage 1, 19 (20.0 %) as stage 2 and 9 (9.5 %) as stage 3. Three patients received RRT starting on $d_{2post\text{-}op}$. Duration of RRT was 3 d in 2 patients and 14 d in 1 patient. The flow of patients over different diagnostic windows for AKI is illustrated in **Figure 1**. The limited extent to which the UO criterion identified AKI patients is illustrated in *Supplemental Figure S3*. Median time to AKI diagnosis was 12.2 h (IQR: 7.2-18.4 h). Subclinical AKI, which was missed by KDIGO, occurred in 5.1 % of the patients **(Figure 2)**.

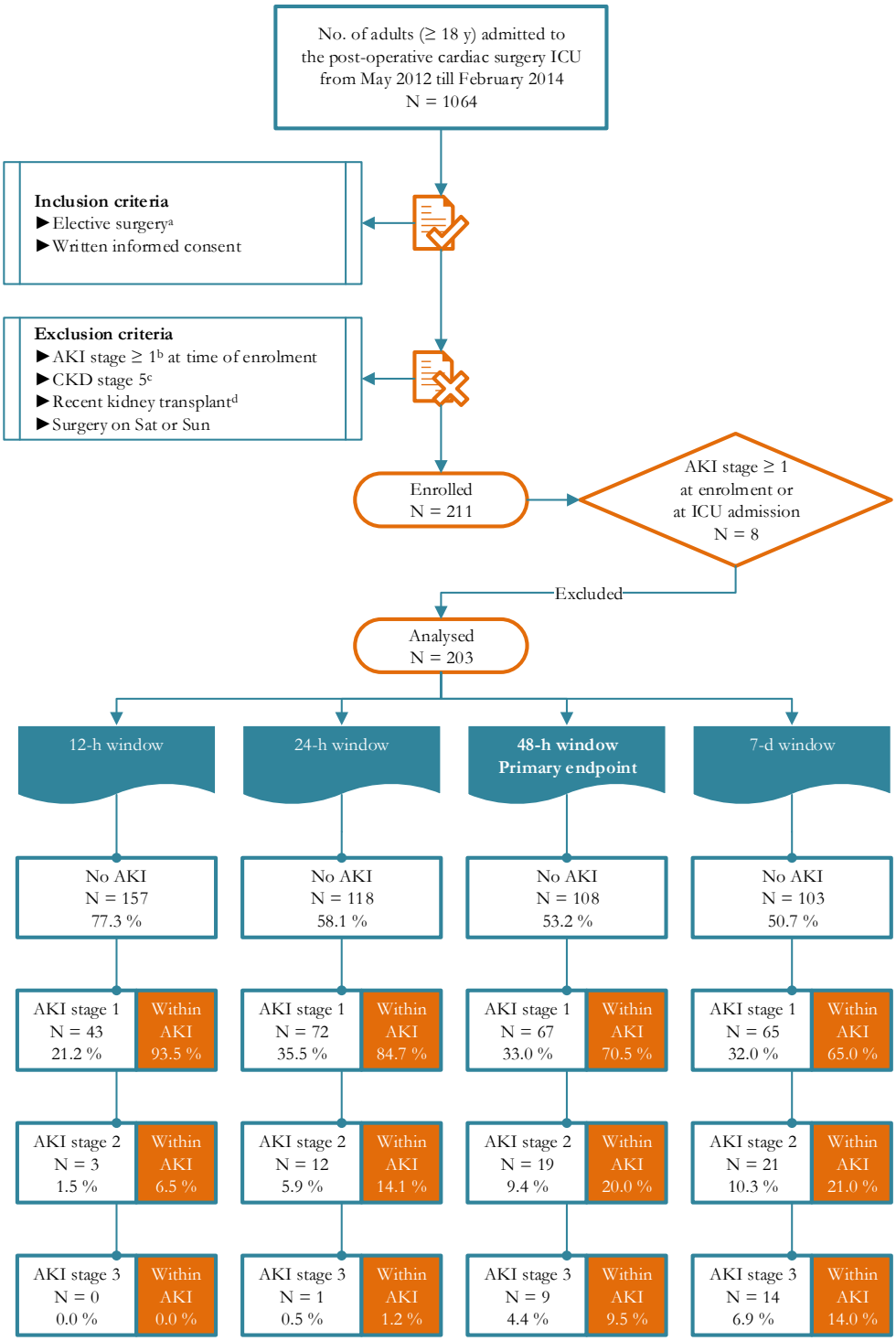

No. of adults (≥ 18 y) admitted to the post-operative cardiac surgery ICU from May 2012 till February 2014
N = 1064
Inclusion criteria
▶Elective surgery[a]
▶Written informed consent
Exclusion criteria
▶AKI stage ≥ 1[b] at time of enrolment
▶CKD stage 5[c]
▶Recent kidney transplant[d]
▶Surgery on Sat or Sun
Enrolled
N = 211
AKI stage ≥ 1 at enrolment or at ICU admission
N = 8
Excluded
Analysed
N = 203
12-h window
24-h window
48-h window
Primary endpoint
7-d window
No AKI
N = 157
77.3 %
No AKI
N = 118
58.1 %
No AKI
N = 108
53.2 %
No AKI
N = 103
50.7 %
AKI stage 1
N = 43
21.2 %
Within AKI
93.5 %
AKI stage 1
N = 72
35.5 %
Within AKI
84.7 %
AKI stage 1
N = 67
33.0 %
Within AKI
70.5 %
AKI stage 1
N = 65
32.0 %
Within AKI
65.0 %
AKI stage 2
N = 3
1.5 %
Within AKI
6.5 %
AKI stage 2
N = 12
5.9 %
Within AKI
14.1 %
AKI stage 2
N = 19
9.4 %
Within AKI
20.0 %
AKI stage 2
N = 21
10.3 %
Within AKI
21.0 %
AKI stage 3
N = 0
0.0 %
Within AKI
0.0 %
AKI stage 3
N = 1
0.5 %
Within AKI
1.2 %
AKI stage 3
N = 9
4.4 %
Within AKI
9.5 %
AKI stage 3
N = 14
6.9 %
Within AKI
14.0 %

Figure 1 | Flow diagram of patient enrolment and primary endpoint analysis
[a]Planned ≥ 4 h in advance
[b]KDIGO definitions for the diagnosis and staging of AKI, which are based on SCr and UO [11]
[c]KDOQI definitions for the diagnosis and staging of CKD [144]
[d]≤ 3 mo before
Abbreviations: AKI acute kidney injury, CKD chronic kidney disease, ICU intensive care unit, KDIGO Kidney Disease | Improving Global Outcomes, KDOQI Kidney Disease Outcomes Quality Initiative, No. number, SCr serum creatinine, UO urine output

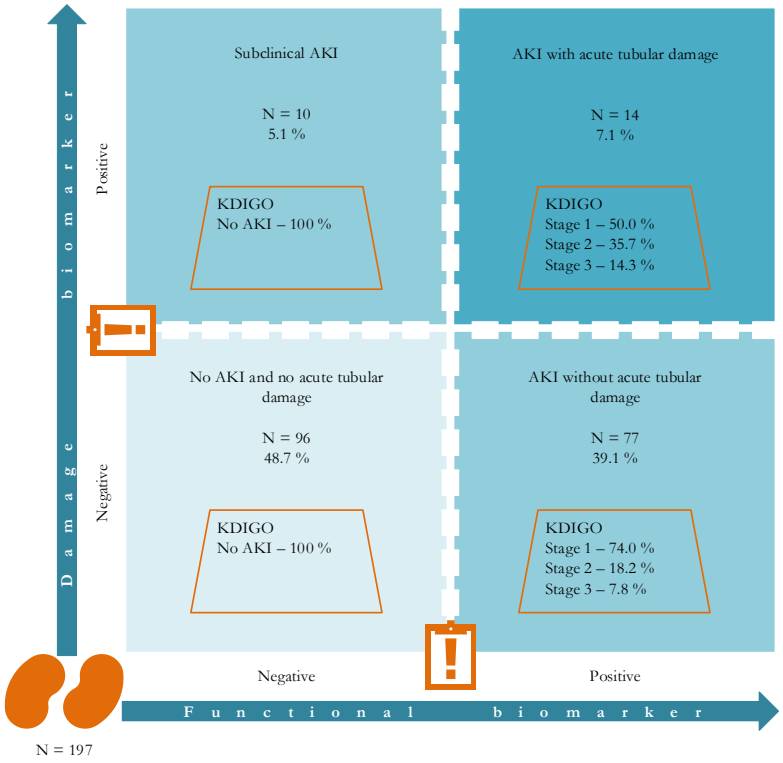


Figure 2 | Combining functional and damage biomarkers simultaneously to delineate the spectrum of acute kidney injury
Patients of the primary analysis cohort (n = 203) who had no $UNGAL_{t1}$ or $UNGAL_{t3}$ concentration available were excluded. Missing $UNGAL_{t1}$ occurred in 5 patients, and missing $UNGAL_{t3}$ in 1 patient, resulting in a total of 197 patients. Following the recommendations of de Geus et al., acute tubular damage was defined as a CSA-NGAL score of 2 or greater, i.e. either as $UNGAL_{t1}$ or $UNGAL_{t3} \geq 150$ ng/ml or as $\Delta UNGAL_{t3\text{-}t1} > 100$ ng/ml with $UNGAL_{t3} \geq 125$ ng/ml. Subclinical AKI was defined when there was acute tubular damage (according to the 'de Geus criteria') and absence of AKI according to the KDIGO definition. In this way 84.6 % of AKI in our specific cohort (i.e., 77/[77+14]) was classified as AKI without acute tubular damage. Further, we found that when there was acute tubular damage, 41.7 % of these patients had no AKI according to the KDIGO definition (i.e., subclinical AKI).
Abbreviations: AKI acute kidney injury, CSA cardiac surgery-associated, KDIGO Kidney Disease | Improving Global Outcomes, t1 time of intensive care unit admission, t3 4 h after intensive care unit admission, UNGAL urinary neutrophil gelatinase-associated lipocalin

Table 1 summarizes the characteristics of the patients and procedures at baseline. Compared with patients without AKI, patients who developed AKI within 48 h after t1 were older; had a higher body mass index **(BMI)**; a lower eGFR; a higher prevalence of diabetes mellitus **(DM)** type 2; a higher predicted operative mortality, which was estimated by the simple additive European system for cardiac operative risk evaluation **(EuroSCORE)**; more combined surgical procedures; and a higher prevalence of diuretic treatment at home.

Table 1 | Characteristics of the patients and procedures at baseline as well as short-term patient outcomes

	Total	AKI stage ≥ 1 within 48 h[a]	No AKI within 48 h[a]	P value
Number of patients	203 (100 %) [98.1-100 %]	95 (46.8 %) [40.1-53.7 %]	108 (53.2 %) [46.3-59.9 %]	NA
Characteristics				
Male sex	133 (65.5 %) [58.7-71.7 %]	65 (68.4 %) [58.5-76.9 %]	68 (63.0 %) [53.6-71.5 %]	0.461
White race	202 (99.5 %) [97.3-99.9 %]	95 (100 %) [96.1-100 %]	107 (99.1 %) [94.9-99.8 %]	1.000
Age (y)[b]	70.0 (61.0-76.0)	74.0 (65.0-80.0)	67.0 (58.0-75.0)	< 0.001
BMI	27 (24-29)	27 (25-31)	26 (23-29)	0.004
Medical history				
Reference renal function				
SCr (mg/dl)	0.90 (0.75-1.05)	0.99 (0.81-1.16)	0.82 (0.70-0.95)	< 0.001
$eGFR_{CKD-EPI}$ (ml/min/1.73 m^2)	82 (64-94)	73 (54-85)	87 (77-97)	< 0.001
DM				0.025
Type 1	2 (1.0 %) [0.3-3.5 %]	2 (2.1 %) [0.6-7.4 %]	0 (0.0 %) [0.0-3.4 %]	
Type 2	46 (22.7 %) [17.4-28.9 %]	28 (29.5 %) [21.2-39.3 %]	18 (16.7 %) [10.8-24.8 %]	
No DM	155 (76.4 %) [70.1-81.7 %]	65 (68.4 %) [58.5-76.9 %]	90 (83.3 %) [75.2-89.2 %]	
Heart failure				0.252
NYHA class I	145 (71.4 %) [64.9-77.2 %]	64 (67.4 %) [57.4-76.0 %]	81 (75.0 %) [66.1-82.2 %]	
NYHA class II	36 (17.7 %) [13.1-23.6 %]	17 (17.9 %) [11.5-26.8 %]	19 (17.6 %) [11.6-25.8 %]	
NYHA class III	20 (9.9 %) [6.5-14.7 %]	12 (12.6 %) [7.4-20.8 %]	8 (7.4 %) [3.8-13.9 %]	
NYHA class IV	2 (1.0 %) [0.3-3.5 %]	2 (2.1 %) [0.6-7.4 %]	0 (0.0 %) [0.0-3.4 %]	
Clinical examination				
Blood pressure (mmHg)				
Systolic	134 (122-149)	132 (122-150)	134 (120-149)	0.704
Diastolic	72 (64-78)	72 (63-78)	71 (66-78)	0.619
Mean	93 (85-101)	92 (84-102)	93 (86-100)	0.734
Heart rhythm				0.074
Atrial fibrillation	16 (7.9 %) [4.9-12.4 %]	11 (11.6 %) [6.6-19.6 %]	5 (4.6 %) [2.0-10.4 %]	
Normal sinus	187 (92.1 %)	84 (88.4 %)	103 (95.4 %)	

	Total	AKI stage ≥ 1 within 48 h[a]	No AKI within 48 h[a]	P value
rhythm	[87.6-95.1 %]	[80.4-93.4 %]	[89.6-98.0 %]	
Heart rate in normal sinus rhythm (bpm) N = 187	69 (61-79)	70 (62-81)	69 (60-76)	0.143
Distribution of ejection fraction				0.531
≤ 20 %	5 (2.5 %) [1.1-5.6 %]	4 (4.2 %) [1.6-10.3 %]	1 (0.9 %) [0.2-5.1 %]	
21-30 %	3 (1.5 %) [0.5-4.3 %]	2 (2.1 %) [0.6-7.4 %]	1 (0.9 %) [0.2-5.1 %]	
31-50 %	33 (16.3 %) [11.8-22.0 %]	16 (16.8 %) [10.6-25.6 %]	17 (15.7 %) [10.1-23.8 %]	
> 50 %	119 (58.6 %) [51.7-65.2 %]	55 (57.9 %) [47.8-67.3 %]	64 (59.3 %) [49.8-68.1 %]	
Index surgical procedure				
EuroSCORE	5 (3-8)	6 (4-9)	5 (2-7)	0.004
Type of cardiac surgical procedure				0.005
Isolated CABG	93 (45.8 %) [39.1-52.7 %]	34 (35.8 %) [26.9-45.8 %]	59 (54.6 %) [45.2-63.7 %]	
Isolated valve repair or replacement	55 (27.1 %) [21.4-33.6 %]	28 (29.5 %) [21.2-39.3 %]	27 (25.0 %) [17.8-33.9 %]	
CABG and valve repair or replacement	34 (16.7 %) [12.2-22.5 %]	25 (26.3 %) [18.5-36.0 %]	9 (8.3 %) [4.4-15.1 %]	
Aortic root	12 (5.9 %) [3.4-10.0 %]	5 (5.3 %) [2.3-11.7 %]	7 (6.5 %) [3.2-12.8 %]	
Other	9 (4.4 %) [2.3-8.2 %]	3 (3.2 %) [1.1-8.9 %]	6 (5.6 %) [2.6-11.6 %]	
ECC				0.516
Yes	179 (88.2 %) [83.0-91.9 %]	82 (86.3 %) [78.0-91.8 %]	97 (89.8 %) [82.7-94.2 %]	
No	24 (11.8 %) [8.1-17.0 %]	13 (13.7 %) [8.2-22.0 %]	11 (10.2 %) [5.8-17.3 %]	
Duration of ECC (min) N = 179	91.5 (70.8-123.3)	92.0 (70.3-131.3)	91.5 (70.3-117.8)	0.527
Priming volume of ECC pump (ml) N = 179	1300 (1200-1500)	1300 (1200-1500)	1300 (1150-1400)	0.246
Duration of aortic clamp during ECC (min) N = 179	56.0 (42.8-82.0)	62.0 (45.0-88.0)	55.0 (40.5-74.5)	0.174
Duration of ischemia during ECC (min) N = 179	54.0 (39.0-78.0)	61.0 (43.0-81.0)	52.0 (37.8-70.5)	0.164
Duration of surgery (h)	4.6 (3.9-5.2)	4.7 (3.9-5.3)	4.5 (3.9-5.1)	0.196
IABP peri-operatively	7 (3.4 %) [1.7-6.9 %]	2 (2.1 %) [0.6-7.4 %]	5 (4.6 %) [2.0-10.4 %]	0.452
Medication				
Statins	127 (62.6 %) [55.7-68.9 %]	63 (66.3 %) [56.3-75.0 %]	64 (59.3 %) [49.8-68.1 %]	0.313
ACE inhibitors	56 (27.6 %) [21.9-34.1 %]	28 (29.5 %) [21.2-39.3 %]	28 (25.9 %) [18.6-34.9 %]	0.638
ARBs	7 (3.4 %)	6 (6.3 %)	1 (0.9 %)	0.052

	Total	AKI stage ≥ 1 within 48 h[a]	No AKI within 48 h[a]	P value
	[1.7-6.9 %]	[2.9-13.1 %]	[0.2-5.1 %]	
Diuretics	51 (25.1 %) [19.7-31.5 %]	36 (37.9 %) [28.8-47.9 %]	15 (13.9 %) [8.6-21.7 %]	< 0.001
NSAIDs	4 (2.0 %) [0.8-5.0 %]	1 (1.1 %) [0.2-5.7 %]	3 (2.8 %) [0.9-7.9 %]	0.624
Corticosteroids	13 (6.4 %) [3.8-10.6 %]	8 (8.4 %) [4.3-15.7 %]	5 (4.6 %) [2.0-10.4 %]	0.390
Tacrolimus	0 (0.0 %) [0.0-1.9 %]	0 (0.0 %) [0.0-3.9 %]	0 (0.0 %) [0.0-3.4 %]	NA
Cyclosporine	2 (1.0 %) [0.3-3.5 %]	2 (2.1 %) [0.6-7.4 %]	0 (0.0 %) [0.0-3.4 %]	0.218
Aminoglycosides	3 (1.5 %) [0.5-4.3 %]	1 (1.1 %) [0.2-5.7 %]	2 (1.9 %) [0.5-6.5 %]	1.000
Corticosteroids intra-operatively	0 (0.0 %) [0.0-1.9 %]	0 (0.0 %) [0.0-3.9 %]	0 (0.0 %) [0.0-3.4 %]	NA
Iodinated contrast ≤ 72 h before surgery	37 (18.2 %) [13.5-24.1 %]	14 (14.7 %) [9.0-23.2 %]	23 (21.3 %) [14.6-29.9 %]	0.275
Outcomes				
RRT in ICU	3 (1.5 %) [0.5-4.3 %]	3 (3.2 %) [1.1-8.9 %]	0 (0.0 %) [0.0-3.4 %]	0.101
ICU LOS (d)	1 (1-3)	2 (1-3)	1 (1-2)	< 0.001
Hospital LOS (d)	12 (9-16)	13 (10-20)	10 (9-13)	< 0.001

Data represent number (proportion) [95 % CI of proportion] for categorical variables and median (IQR) for continuous variables.

[a]KDIGO definitions for the diagnosis and staging of AKI, which are based on SCr and UO

[b]Determined at the day of surgery

Abbreviations: ACE angiotensin-converting enzyme, AKI acute kidney injury, ARB angiotensin-II receptor blocker, BMI body mass index, bpm beats per minute, CABG coronary artery bypass grafting, CI confidence interval, CKD-EPI Chronic Kidney Disease Epidemiology Collaboration, DM diabetes mellitus, ECC extracorporeal circulation, eGFR estimated glomerular filtration rate, EuroSCORE European System for Cardiac Operative Risk Evaluation, h hour, IABP intra-aortic balloon pump, ICU intensive care unit, IQR interquartile range, KDIGO Kidney Disease | Improving Global Outcomes, LOS length of stay, min minute, no. number, NSAID nonsteroidal anti-inflammatory drug, NYHA New York Heart Association, RRT renal replacement therapy, SCr serum creatinine, UO urine output

Peri-operative characteristics of the patients and procedures are provided in *Supplemental Table S4*. In comparison with patients without AKI, patients who developed AKI within 48 h after t1 had a higher SOFA score; a higher white blood cell count; a higher serum C-reactive protein concentration; a more positive fluid balance; a higher number of plasma units that were transfused; and a higher prevalence of vasopressor, milrinone and diuretic treatment.

AKI patients had a longer ICU and hospital LOS **(Table 1)**.

INCIDENCE OF THE SECONDARY ENDPOINT

In the secondary endpoint analysis, 1 patient who already had AKI stage ≥ 2 at t0 was excluded, resulting in a total number of 210 patients for analysis. Within 12 h after t1, 5 patients (2.4 %) had developed AKI stage ≥ 2: all 5 (100 %) were classified as stage 2. The flow of patients over different diagnostic windows for AKI stage ≥ 2 is illustrated in *Supplemental Figure S4*.

BIOMARKER PERFORMANCES FOR PREDICTION OF THE PRIMARY ENDPOINT

In the early post-operative period, only the functional biomarker SCr was a good predictor of AKI within 48 h after t1, showing the highest AUC-ROC at t3 (0.792; 95 % CI: 0.728-0.847) **(Figure 3)**. The information in Figure 3 is summarized in numerical format in *Supplemental Tables S5B-D*, including also the performances of the urinary renal stress or damage biomarkers corrected for urine dilution by using the ratio to UCr. *Table S5A* reports the performances of the biomarkers measured at t0.

BIOMARKER PERFORMANCES FOR PREDICTION OF THE SECONDARY ENDPOINT

In the early post-operative period, SCHI3L1 combined with a urinary renal stress or damage biomarker, either UCHI3L1 or UNGAL, was a good predictor of AKI stage ≥ 2 within 12 h after t1, showing the highest AUC-ROC at t2 when combined with UCHI3L1 (0.773; 95 % CI: 0.708-0.829) and at t3 when combined with UNGAL (0.774; 95 % CI: 0.710-0.830). However, the functional biomarkers SCr and ΔSCr_{tx-t0} outperformed these combinations with good to excellent performances, showing the highest AUC-ROC at t3 (0.857 for SCr; 95 % CI: 0.801-0.902; 0.938 for ΔSCr_{tx-t0}; 95 % CI: 0.860-1.000) **(Figure 3)**. The information in Figure 3 is summarized in numerical format in *Supplemental Tables S5B-D*, including also the performances of the urinary renal stress or damage biomarkers corrected for urine dilution by using the ratio to UCr. *Table S5A* reports the performances of the biomarkers measured at t0.

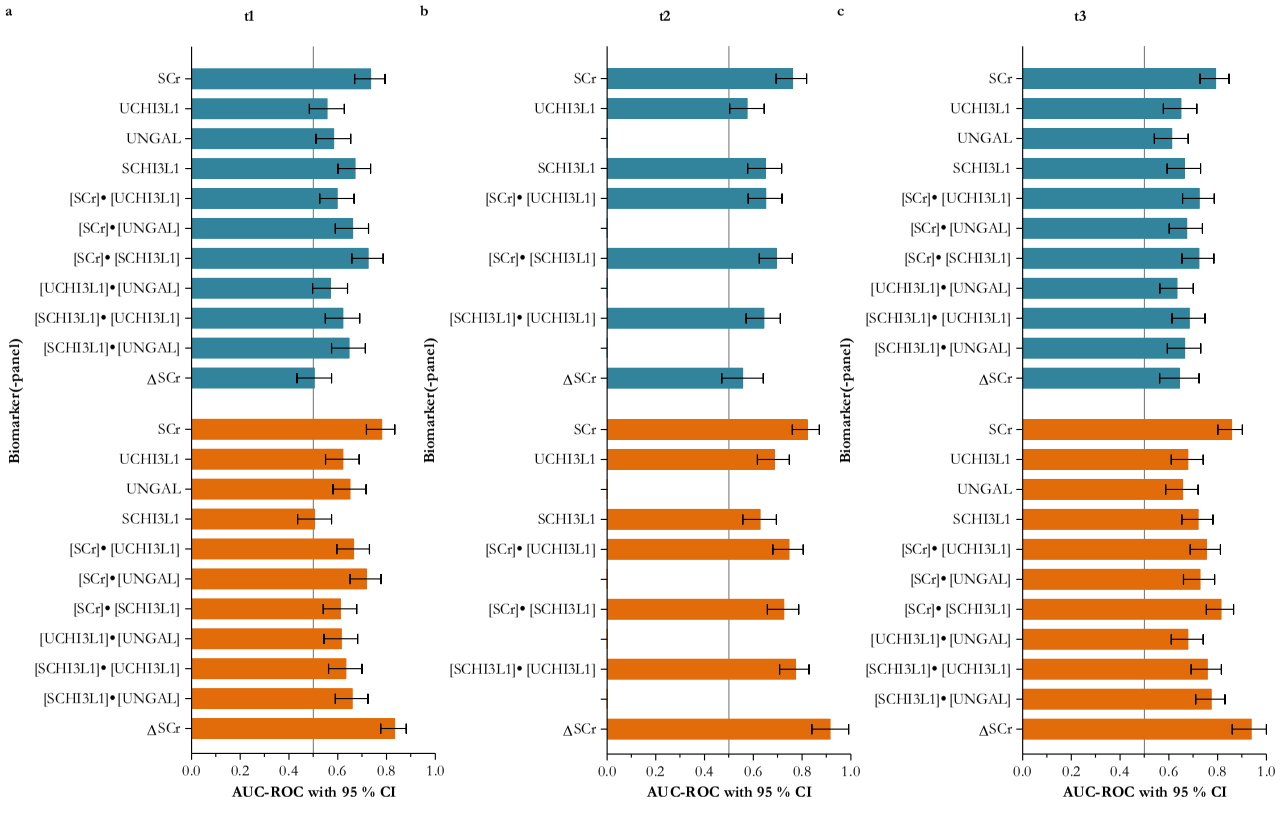


Figure 3 | Biomarker performances for prediction of acute kidney injury Green boxes correspond with the primary endpoint (i.e., AKI stage ≥ 1 within 48 h after t1), orange boxes with the secondary endpoint (i.e., AKI stage ≥ 2 within 12 h after t1). ΔSCr represents ΔSCr_{tx-t0}, which is the absolute change in SCr between SCr_{tx} and SCr_{t0}. Biomarkers were measured ***(a)*** at ICU admission (t1), ***(b)*** 2 h after ICU admission (t2), and ***(c)*** 4 h after ICU admission (t3).

Abbreviations: AKI acute kidney injury, ICU intensive care unit, SCHI3L1 serum chitinase 3-like protein 1, SCr serum creatinine, UCHI3L1 urinary chitinase 3-like protein 1, UNGAL urinary neutrophil gelatinase-associated lipocalin

V. Discussion

Additions to the published paper are marked in italic font.

We found that in adult patients who underwent elective cardiac surgery, UCHI3L1 had inadequate predictive value for CSA-AKI. This was also true for the well-known tubular damage biomarker UNGAL. In contrast, more frequent assessment of the functional biomarker SCr in the early post-operative ICU period (first 4 h) had good to excellent predictive value for CSA-AKI.

Similar to others, our ICU routinely measures SCr at t1 and either around t4 (morning patient) or t5 (afternoon patient) in the early post-operative period. However, with ±50 % of AKI diagnosed before t5, of which ±50 % before t4, our study highlights the importance of more frequent SCr assessment in the first 4 h. This aids in early AKI diagnosis and could also reveal some cases of rapid reversal of AKI (i.e., "complete reversal of AKI by KDIGO criteria within 48 h of AKI onset" [47]). These findings are in accordance with those of a small retrospective study (n = 29) by Maciel et al. [197].

Novel findings of our study are the good to excellent performances of ΔSCr_{t1-t0}, ΔSCr_{t2-t0} and ΔSCr_{t3-t0} for the prediction of AKI stage ≥ 2 within 12 h after t1. In accordance with these results, measurement of very early post-operative SCr changes, either absolute [187] or relative [198, 199], in adult [187, 198] and paediatric [199] cardiac surgery patients has been reported to provide prognostic utility for a subsequent diagnosis of AKI. However, each of these three studies was restricted by some of the following limitations: AKI was not defined according to KDIGO, including oliguric criteria; more severe AKI was not included as endpoint; SCr was less frequently assessed in the early post-operative period; and AUC-ROC analysis was not reported. The simultaneous utilization of functional and damage biomarkers, as in the CSA-NGAL score [195], permits to delineate the spectrum of AKI [133]. In this way 84.6 % of AKI in our specific cohort (i.e., 77/[77+14]) was classified as AKI without acute tubular damage, which can explain the inadequate performance of the urinary renal stress or damage biomarkers UCHI3L1 and

UNGAL for prediction of AKI stage ≥ 2 within 12 h after t1. *Further, we found that when there was acute tubular damage, 41.7 % of these patients had no AKI according to the KDIGO definition (i.e., subclinical AKI).*

The capacity of the kidneys to increase baseline GFR under physiological or pathological kidney stress is described by the renal functional reserve of the glomerular function **(RFR-G)** *(Supplemental Figure S5)* [200]. In congestive heart failure, CKD and AKI, utilization of the RFR-G allows to replace (in part) the lost function, maintaining a normal whole-organ baseline GFR until ±50 % of the nephrons are non-functional. The presence of both a normal RFR-G and a normal functioning nephron mass guarantees a low renal frailty or susceptibility [201]. Compared with the general ICU cohort of our previous AKI biomarker study [193], the patients in this study were older (median 70.0 vs. 60.0 y), more often diabetic (23.6 vs. 7.2 %), had a higher BMI (median 27 vs. 24) and a lower baseline eGFR (median 82 vs. 102 ml/min/1.73 m^2), prompting the hypothesis that the patients in this study had a lower RFR-G or a lower functioning nephron mass. *We further hypothesize that this lower RFR-G or lower functioning nephron mass decreased the incidence of subclinical AKI in this population by increasing the incidence of AKI with acute tubular damage.*

Our study is not the first to report inadequate diagnostic ability of UNGAL for moderate to severe CSA-AKI [192]. In their multicentre high-risk cohort with 21 % emergent surgeries, Parikh et al. reported an AUC-ROC of 0.67 (standard error: 0.04) for the prediction of AKI defined by receipt of RRT or doubling of SCr [168]. Interestingly, Koyner et al. found an AUC-ROC of 0.88 (95 % CI: 0.73-0.99) for the prediction of AKI AKIN stage 3 in a cohort comparable to ours [202], indicating the importance of the severity of AKI in the diagnostic ability of UNGAL.

Our study has several limitations. First, it is limited in being a single-centre study in elective cardiac surgery patients. However, baseline characteristics and the incidence of AKI suggest that our population is representative of cardiac surgery ICUs in developed countries. Second, our study is further limited by the relatively low incidence of AKI stage ≥ 2 within 12 h after t1

(secondary endpoint). Both the fact that we included only elective surgeries and the implemented narrow diagnostic window contribute to this constraint. As such, these biomarker data need to be further validated in larger multicentre prospective studies. Third, we did not measure the panel of G1 cell-cycle arrest biomarkers [TIMP-2]•[IGFBP7] [136], because it was not available at the start of our study.

VI. Conclusions

In summary, we demonstrated that more frequent assessment of the functional biomarker SCr in the early post-operative ICU period (first 4 h) after elective cardiac surgery in adult patients had good to excellent predictive value for CSA-AKI, indicating that routine SCr assessment must become more frequent in order to detect AKI more early. We found that AKI was predominantly without acute tubular damage in this cohort, which may explain the inadequate predictive value for CSA-AKI of the urinary renal stress or damage biomarkers UCHI3L1 and UNGAL. *Further, we found that when there was acute tubular damage, more than 40 % of these patients had no AKI according to the KDIGO definition (i.e., subclinical AKI).* These results need to be further validated in larger multicentre prospective studies.

VII. Supplemental Material

SUPPLEMENTAL TABLES

Table S1 | STROBE statement – checklist of items that should be included in the reports of cohort studies [143]

	Item No.	STROBE recommendation	Fulfilled
Title and abstract			
	1	(a) Indicate the study's design with a commonly used term in the title or the abstract	Yes
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found	Yes
Introduction			
Background / rationale	2	Explain the scientific background and rationale for the investigation being reported	Yes
Objectives	3	State specific objectives, including any pre-specified hypotheses	Yes
Methods			
Study design	4	Present key elements of study design early in the paper	Yes
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	Yes
Participants	6	(a) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	Yes
		(b) For matched studies, give matching criteria and number of exposed and unexposed	NA
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	Yes
Data sources / measurement	8[a]	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	Yes
Bias	9	Describe any efforts to address potential sources of bias	Yes
Study size	10	Explain how the study size was arrived at	Yes
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	Yes
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding	Yes
		(b) Describe any methods used to examine subgroups and interactions	Yes
		(c) Explain how missing data were addressed	Yes
		(d) If applicable, explain how loss to follow-up was addressed	Yes
		(e) Describe any sensitivity analyses	NA
Results			
Participants	13[a]	(a) Report numbers of individuals at each stage of study – e.g., numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	Yes
		(b) Give reasons for non-participation at each stage	Yes
		(c) Consider use of a flow diagram	Yes
Descriptive data	14[a]	(a) Give characteristics of study participants (e.g., demographic, clinical, social) and information on exposures and potential confounders	Yes
		(b) Indicate number of participants with missing data for each variable of interest	Yes
		(c) Summarize follow-up time (e.g., average and total amount)	Yes
Outcome data	15[a]	Report numbers of outcome events or summary measures over time	Yes
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (e.g., 95% confidence interval). Make clear which confounders were adjusted for and why they were included	Yes

	Item No.	STROBE recommendation	Fulfilled
		(b) Report category boundaries when continuous variables were categorized	NA
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	NA
Other analyses	17	Report other analyses done - e.g., analyses of subgroups and interactions, and sensitivity analyses	Yes
Discussion			
Key results	18	Summarize key results with reference to study objectives	Yes
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	Yes
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	Yes
Generalizability	21	Discuss the generalizability (external validity) of the study results	Yes
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	Yes

[a]Give information separately for exposed and unexposed groups

Abbreviations: NA not applicable, No. number, STROBE STrengthening the Reporting of OBservational studies in Epidemiology

Table S2 | Data collected in the clinical research file

I.	**Patient information**	
A.	Study identification	A / □□□
B.	Date of birth	□□ / □□ / □□□□
C.	Sex	□ Male or □ Female
D.	Race	□ Caucasian or □ Black
E.	Smoking status	□ Current smoker or □ Ex-smoker or □ Non-smoker
•	Smoking history: cumulative number of pack-years[a]	▬ Textbox
F.	Alcohol use	□ Yes or □ No
•	Quantity of alcohol intake	▬ Textbox
G.	Weight (kg)	▬ Textbox
H.	Length (cm)	▬ Textbox
I.	BMI	▬ Textbox
II.	**Medical data**	
A.	Medical history	
1.	NYHA class	□ I or □ II or □ III or □ IV
2.	DM	□ Type 1 or □ Type 2 or □ No DM
•	Therapy for DM	□ Insulin therapy or □ Therapy per os
3.	Other comorbidities	▬ Textbox
4.	Pre-operative medication list	▬ Textbox
B.	Admission diagnosis	
1.	Date of hospital admission	□□ / □□ / □□□□
•	Diagnosis at hospital admission	▬ Textbox
2.	Date of ICU admission	□□ / □□ / □□□□
•	Diagnosis at ICU admission	▬ Textbox
C.	Baseline registrations	
1.	Blood pressure (mmHg; highest)	
i.	Systolic / Diastolic	▬ Textbox
ii.	Mean	▬ Textbox
2.	Heart rhythm	□ Atrial fibrillation or □ Normal sinus rhythm
3.	Heart rate in normal sinus rhythm (bpm)	▬ Textbox
4.	Ejection fraction (%)	
i.	Pre-operative	▬ Textbox

ii.	Post-operative	▬ Textbox
5.	Fractional shortening (%)	
i.	Pre-operative	▬ Textbox
ii.	Post-operative	▬ Textbox
6.	Reference SCr	▬ Textbox
7.	Reference eGFRCKD-EPI	▬ Textbox
8.	Medication	
i.	Statins	□ Yes or □ No
ii.	ACE inhibitors	□ Yes or □ No
iii.	ARBs	□ Yes or □ No
iv.	Diuretics	□ Yes or □ No
v.	NSAIDs	□ Yes or □ No
vi.	Corticosteroids	□ Yes or □ No
vii.	Tacrolimus	□ Yes or □ No
viii.	Cyclosporine	□ Yes or □ No
ix.	Aminoglycosides	□ Yes or □ No
x.	Iodinated contrast pre-operatively	□ Yes or □ No
•	Date	□□ / □□ / □□□□
xi.	Corticosteroids intra-operatively	□ Yes or □ No
D.	Index surgical procedure	
1.	EuroSCORE	▬ Textbox
2.	Type of cardiac surgical procedure	▬ Textbox
3.	Duration of surgery (h)	▬ Textbox
4.	IABP peri-operatively	□ Yes or □ No
5.	ECC data	
i.	Mean blood pressure on pump	▬ Textbox
ii.	Diuretics on pump	□ Yes or □ No
iii.	Diuresis on pump (ml)	▬ Textbox
iv.	Priming volume of pump (ml)	▬ Textbox
v.	Haematocrit (%)	
•	Before pump	▬ Textbox
•	After pump	▬ Textbox
vi.	SCr (mg/dl)	
•	Before pump	▬ Textbox
•	After pump	▬ Textbox
vii.	Duration of ECC (min)	▬ Textbox
viii.	Duration of aortic clamp (min)	▬ Textbox
ix.	Duration of ischemia (min)	▬ Textbox
E.	Study follow-up (to be filled in for dsurgery, d1post-op and d2post-op)	
1.	RRT	□ Yes or □ No
•	Date	□□ / □□ / □□□□
2.	SOFA score	▬ Textbox
3.	WBC count (103/µl)	▬ Textbox
4.	Serum CRP (mg/l)	▬ Textbox
5.	Fluid balance	▬ Textbox
•	Total fluid IN (ml)	▬ Textbox
•	Total fluid OUT (ml)	▬ Textbox
6.	Transfusions	
i.	Whole blood	□ Yes or □ No
•	Number of units transfused	▬ Textbox
ii.	Plasma	□ Yes or □ No
•	Number of units transfused	▬ Textbox
iii.	Platelets	□ Yes or □ No
•	Number of units transfused	▬ Textbox
7.	Medication	
i.	Milrinone (PDE3 inhibitor)	□ Yes or □ No
ii.	Vasopressor(s)	□ Yes or □ No
•	Generic name(s)	▬ Textbox
iii.	Antibiotic(s)	□ Yes or □ No
•	Generic name(s)	▬ Textbox
iv.	Statins	□ Yes or □ No

v.	ACE inhibitors	□ Yes or □ No
vi.	ARBs	□ Yes or □ No
vii.	Diuretics	□ Yes or □ No
viii.	Tacrolimus	□ Yes or □ No
ix.	Cyclosporine	□ Yes or □ No
x.	Iodinated contrast post-operatively	□ Yes or □ No

[a]A pack-year is defined as twenty cigarettes smoked every day for one year

Abbreviations: ACE angiotensin-converting enzyme, ARB angiotensin-II receptor blocker, BMI body mass index, bpm beats per minute, CKD-EPI Chronic Kidney Disease Epidemiology Collaboration, CRP C-reactive protein, DM diabetes mellitus, ECC extracorporeal circulation, eGFR estimated glomerular filtration rate, EuroSCORE European System for Cardiac Operative Risk Evaluation, IABP intra-aortic balloon pump, ICU intensive care unit, min minute, NSAID non-steroidal anti-inflammatory drug, NYHA New York Heart Association, PDE3 phosphodiesterase 3, post-op post-operatively, RRT renal replacement therapy, SCr serum creatinine, SOFA Sepsis-related Organ Failure Assessment, WBC white blood cell

Table S3 | Dilution of serum and urine samples for the initial measurement of CHI3L1 by enzyme-linked immunosorbent assay

Time point of the study	Estimated dilution for serum sample	Estimated dilution for urine sample
t0	1/100	1/2
t1	1/100	1/2
t2	1/200	1/4
t3	1/200	1/4
t4	1/200	1/4
t5	1/500	1/4
t6	1/500	1/4
t7	1/500	1/4

Abbreviations: CHI3L1 chitinase 3-like protein 1

Table S4 | Characteristics of the patients and procedures peri-operatively

	Day of surgery				First post-operative day				Second post-operative day			
	Total	AKI stage ≥ 1 within 48 h[a]	No AKI within 48 h[a]	P value	Total	AKI stage ≥ 1 within 48 h[a]	No AKI within 48 h[a]	P value	Total	AKI stage ≥ 1 within 48 h[a]	No AKI within 48 h[a]	P value
Number of patients	203 (100 %) [98.1-100 %]	95 (46.8 %) [40.1-53.7 %]	108 (53.2 %) [46.3-59.9 %]	NA	203 (100 %) [98.1-100 %]	95 (46.8 %) [40.1-53.7 %]	108 (53.2 %) [46.3-59.9 %]	NA	203 (100 %) [98.1-100 %]	95 (46.8 %) [40.1-53.7 %]	108 (53.2 %) [46.3-59.9 %]	NA
Characteristics												
SOFA score	9 (8-10)	9 (8-10)	8 (7-9)	< 0.001	5 (3-7)	6 (4-8)	4 (3-6)	< 0.001	3 (2-4)	4 (3-6)	2 (1-3)	< 0.001
WBC count ($10^3/\mu l$)	10.7 (8.4-12.9)	10.6 (8.0-13.4)	10.7 (8.5-12.9)	0.958	12.4 (9.9-14.9)	12.7 (11.0-15.8)	11.5 (9.3-14.9)	0.032	12.0 (10.0-14.8)	12.9 (10.3-15.9)	11.5 (9.7-14.4)	0.012
Serum CRP (mg/l)	9.5 (4.0-18.0)	9.8 (5.0-16.5)	9.3 (3.4-18.0)	0.591	67.0 (37.6-118.7)	89.0 (45.5-137.4)	56.0 (36.0-95.8)	0.001	198.4 (127.3-246.3)	205.0 (156.8-265.4)	143.6 (117.6-230.8)	0.043
Fluid balance (ml)	1014 (598-1460)	1269 (827-1613)	797 (432-1235)	< 0.001	568 (-226 to 1518)	1105 (190-1977)	291 (-512 to 1084)	< 0.001	299 (-597 to 934)	549 (-186 to 1206)	26 (-880 to 781)	0.004
Procedure												
Transfusion(s)												
Whole blood	37 (18.2 %) [13.5-24.1 %]	22 (23.2 %) [15.8-32.6 %]	15 (13.9 %) [8.6-21.7 %]	0.102	13 (6.4 %) [3.8-10.6 %]	8 (8.4 %) [4.3-15.7 %]	5 (4.6 %) [2.0-10.4 %]	0.390	11 (5.4 %) [3.1-9.4 %]	7 (7.4 %) [3.6-14.4 %]	4 (3.7 %) [1.4-9.1 %]	0.354
Plasma	14 (6.9 %) [4.2-11.2 %]	6 (6.3 %) [2.9-13.1 %]	8 (7.4 %) [3.8-13.9 %]	0.789	3 (1.5 %) [0.5-4.3 %]	2 (2.1 %) [0.6-7.4 %]	1 (0.9 %) [0.2-5.1 %]	0.600	3 (1.5 %) [0.5-4.3 %]	3 (3.2 %) [1.1-8.9 %]	0 (0.0 %) [0.0-3.4 %]	0.101
Platelets	11 (5.4 %) [3.1-9.4 %]	8 (8.4 %) [4.3-15.7 %]	3 (2.8 %) [0.9-7.9 %]	0.118	1 (0.5 %) [0.1-2.7 %]	1 (1.1 %) [0.2-5.7 %]	0 (0.0 %) [0.0-3.4 %]	0.468	1 (0.5 %) [0.1-2.7 %]	1 (1.1 %) [0.2-5.7 %]	0 (0.0 %) [0.0-3.4 %]	0.468
Number of units transfused												
Whole blood	2.0 (1.0-3.0)	2.0 (1.0-3.5)	2.0 (1.0-2.0)	0.531	1.0 (1.0-2.0)	1.0 (1.0-2.0)	1.0 (1.0-2.0)	1.000	1.0 (1.0-2.0)	1.0 (1.0-3.0)	1.0 (1.0-1.0)	0.315
Plasma	3.0 (2.0-4.3)	4.5 (3.8-5.3)	2.0 (2.0-2.8)	0.001	1.0[b]	1.5[b]	1.0[b]	1.000	3.0[b]	3.0[b]	NA	NA
Platelets	9.0 (6.0-10.0)	8.5 (6.5-10.0)	10.0[b]	0.921	1.0[b]	1.0[b]	NA	NA	10.0[b]	10.0[b]	NA	NA
Medication												
Vaso-pressors	98 (48.3 %) [41.5-55.1 %]	49 (51.6 %) [41.7-61.4 %]	49 (45.4 %) [36.3-54.8 %]	0.401	92 (45.3 %) [38.6-52.2 %]	48 (50.5 %) [40.6-60.4 %]	44 (40.7 %) [31.9-50.2 %]	0.203	29 (14.3 %) [10.1-19.8 %]	20 (21.1 %) [14.1-30.3 %]	9 (8.3 %) [4.4-15.1 %]	0.015
Milrinone (PDE3 inhibitor)	19 (9.4 %) [6.1-14.2 %]	14 (14.7 %) [9.0-23.2 %]	5 (4.6 %) [2.0-10.4 %]	0.016	21 (10.3 %) [6.9-15.3 %]	15 (15.8 %) [9.8-24.4 %]	6 (5.6 %) [2.6-11.6 %]	0.021	14 (6.9 %) [4.2-11.2 %]	10 (10.5 %) [5.8-18.3 %]	4 (3.7 %) [1.4-9.1 %]	0.093

Statins	4 (2.0 %) [0.8-5.0 %]	1 (1.1 %) [0.2-5.7 %]	3 (2.8 %) [0.9-7.9 %]	0.624	93 (45.8 %) [39.1-52.7 %]	44 (46.3 %) [36.6-56.3 %]	49 (45.4 %) [36.3-54.8 %]	1.000	107 (52.7 %) [45.9-59.5 %]	46 (48.4 %) [38.6-58.3 %]	61 (56.5 %) [47.1-65.5 %]	0.263
ACE inhibitors	1 (0.5 %) [0.1-2.7 %]	0 (0.0 %) [0.0-3.9 %]	1 (0.9 %) [0.2-5.1 %]	1.000	20 (9.9 %) [6.5-14.7 %]	8 (8.4 %) [4.3-15.7 %]	12 (11.1 %) [6.5-18.4 %]	0.639	29 (14.3 %) [10.1-19.8 %]	12 (12.6 %) [7.4-20.8 %]	17 (15.7 %) [10.1-23.8 %]	0.554
ARBs	0 (0.0 %) [0.0-1.9 %]	0 (0.0 %) [0.0-3.9 %]	0 (0.0 %) [0.0-3.4 %]	NA	3 (1.5 %) [0.5-4.3 %]	1 (1.1 %) [0.2-5.7 %]	2 (1.9 %) [0.5-6.5 %]	1.000	3 (1.5 %) [0.5-4.3 %]	1 (1.1 %) [0.2-5.7 %]	2 (1.9 %) [0.5-6.5 %]	1.000
Diuretics	12 (5.9 %) [3.4-10.0 %]	7 (7.4 %) [3.6-14.4 %]	5 (4.6 %) [2.0-10.4 %]	0.553	120 (59.1 %) [52.2-65.6 %]	73 (76.8 %) [67.4-84.2 %]	47 (43.5 %) [34.5-52.9 %]	< 0.001	93 (45.8 %) [39.1-52.7 %]	55 (57.9 %) [47.8-67.3 %]	38 (35.2 %) [26.8-44.6 %]	0.002
Tacro-limus	0 (0.0 %) [0.0-1.9 %]	0 (0.0 %) [0.0-3.9 %]	0 (0.0 %) [0.0-3.4 %]	NA	0 (0.0 %) [0.0-1.9 %]	0 (0.0 %) [0.0-3.9 %]	0 (0.0 %) [0.0-3.4 %]	NA	0 (0.0 %) [0.0-1.9 %]	0 (0.0 %) [0.0-3.9 %]	0 (0.0 %) [0.0-3.4 %]	NA
Cyclo-sporine	0 (0.0 %) [0.0-1.9 %]	0 (0.0 %) [0.0-3.9 %]	0 (0.0 %) [0.0-3.4 %]	NA	2 (1.0 %) [0.3-3.5 %]	2 (2.1 %) [0.6-7.4 %]	0 (0.0 %) [0.0-3.4 %]	0.218	2 (1.0 %) [0.3-3.5 %]	2 (2.1 %) [0.6-7.4 %]	0 (0.0 %) [0.0-3.4 %]	0.218
Amino-glycosides	3 (1.5 %) [0.5-4.3 %]	1 (1.1 %) [0.2-5.7 %]	2 (1.9 %) [0.5-6.5 %]	1.000	3 (1.5 %) [0.5-4.3 %]	1 (1.1 %) [0.2-5.7 %]	2 (1.9 %) [0.5-6.5 %]	1.000	2 (1.0 %) [0.3-3.5 %]	1 (1.1 %) [0.2-5.7 %]	1 (0.9 %) [0.2-5.1 %]	1.000
Iodinated contrast post-operative	0 (0.0 %) [0.0-1.9 %]	0 (0.0 %) [0.0-3.9 %]	0 (0.0 %) [0.0-3.4 %]	NA	1 (0.5 %) [0.1-2.7 %]	1 (1.1 %) [0.2-5.7 %]	0 (0.0 %) [0.0-3.4 %]	0.468	2 (1.0 %) [0.3-3.5 %]	1 (1.1 %) [0.2-5.7 %]	1 (0.9 %) [0.2-5.1 %]	1.000

Data represent number (proportion) [95 % CI of proportion] for categorical variables and median (IQR) for continuous variables.

[a]KDIGO definitions for the diagnosis and staging of AKI, which are based on SCr and UO

[b]Median (no calculation of IQR when n < 4)

Abbreviations: ACE angiotensin converting enzyme, AKI acute kidney injury, ARB angiotensin-II receptor blocker, CI confidence interval, CRP C-reactive protein, IQR interquartile range, KDIGO Kidney Disease: Improving Global Outcomes, no. number, PDE3 phosphodiesterase 3, SCr serum creatinine, SOFA Sepsis-related Organ Failure Assessment, UO urine output, WBC white blood cell

Table S5A | Biomarker performances at t0 for prediction of acute kidney injury

	AKI stage ≥ 2 within 12 h after t1[a]			AKI stage ≥ 1 within 48 h after t1[a]		
	AUC-ROC	Lower limit of 95 % CI	Upper limit of 95 % CI	AUC-ROC	Lower limit of 95 % CI	Upper limit of 95 % CI
[SCHI3L1]•[UCHI3L1]	0.595	0.524	0.663	0.599	0.526	0.668
[SCr]•[SCHI3L1]	0.580	0.510	0.648	0.705	0.637	0.768
[SCr]•[UCHI3L1]	0.669	0.599	0.733	0.582	0.510	0.652
SCHI3L1	0.508	0.438	0.578	0.660	0.590	0.725
UCHI3L1/UCr	0.530	0.459	0.601	0.518	0.445	0.589
UCHI3L1	0.578	0.507	0.647	0.532	0.459	0.603
SCr	0.696	0.629	0.758	0.709	0.641	0.771

[a]KDIGO definitions for the diagnosis and staging of AKI, which are based on SCr and UO

Abbreviations: AKI acute kidney injury, AUC-ROC area under the receiver-operating characteristics curve, CI confidence interval, KDIGO Kidney Disease | Improving Global Outcomes, SCHI3L1 serum chitinase 3-like protein 1, SCr serum creatinine, t0 time after the induction of anaesthesia and before the start of surgery, t1 time of intensive care unit admission, UCHI3L1 urinary chitinase 3-like protein 1, UCr urinary creatinine, UO urine output

Table S5B | Biomarker performances at t1 for prediction of acute kidney injury

	AKI stage ≥ 2 within 12 h after t1[a]			AKI stage ≥ 1 within 48 h after t1[a]		
	AUC-ROC	Lower limit of 95 % CI	Upper limit of 95 % CI	AUC-ROC	Lower limit of 95 % CI	Upper limit of 95 % CI
ΔSCr	0.833	0.776	0.881	0.504	0.433	0.575
[SCHI3L1]•[UNGAL]	0.659	0.590	0.724	0.646	0.575	0.713
[SCHI3L1]•[UCHI3L1]	0.633	0.562	0.699	0.621	0.549	0.690
[UCHI3L1]•[UNGAL]	0.615	0.544	0.682	0.570	0.497	0.640
[SCr]•[SCHI3L1]	0.611	0.541	0.678	0.725	0.658	0.786
[SCr]•[UNGAL]	0.718	0.651	0.779	0.661	0.590	0.726
[SCr]•[UCHI3L1]	0.665	0.596	0.730	0.598	0.526	0.667
SCHI3L1	0.505	0.435	0.575	0.671	0.601	0.735
UNGAL/UCr	0.648	0.578	0.713	0.599	0.528	0.668
UNGAL	0.650	0.581	0.716	0.583	0.511	0.653
UCHI3L1/UCr	0.631	0.561	0.698	0.557	0.484	0.628
UCHI3L1	0.621	0.550	0.688	0.556	0.484	0.627
SCr	0.780	0.718	0.834	0.735	0.669	0.794

[a]KDIGO definitions for the diagnosis and staging of AKI, which are based on SCr and UO

Abbreviations: AKI acute kidney injury, AUC-ROC area under the receiver-operating characteristics curve, CI confidence interval, ΔSCr represents $\Delta SCr_{t1\text{-}t0}$, which is the absolute change in SCr between SCr_{t1} and SCr_{t0}, KDIGO Kidney Disease | Improving Global Outcomes, SCHI3L1 serum chitinase 3-like protein 1, SCr serum creatinine, t0 time after the induction of anaesthesia and before the start of surgery, t1 time of intensive care unit admission, UCHI3L1 urinary chitinase 3-like protein 1, UCr urinary creatinine, UNGAL urinary neutrophil gelatinase-associated lipocalin, UO urine output

Table S5C | Biomarker performances at t2 for prediction of acute kidney injury

	AKI stage ≥ 2 within 12 h after t1[a]			AKI stage ≥ 1 within 48 h after t1[a]		
	AUC-ROC	Lower limit of 95 % CI	Upper limit of 95 % CI	AUC-ROC	Lower limit of 95 % CI	Upper limit of 95 % CI
ΔSCr	0.915	0.840	0.991	0.556	0.471	0.641
[SCHI3L1]•[UCHI3L1]	0.773	0.708	0.829	0.643	0.571	0.711
[SCr]•[SCHI3L1]	0.725	0.658	0.786	0.695	0.625	0.760
[SCr]•[UCHI3L1]	0.746	0.680	0.805	0.651	0.579	0.718
SCHI3L1	0.628	0.557	0.695	0.649	0.577	0.716
UCHI3L1/UCr	0.662	0.593	0.726	0.574	0.502	0.644
UCHI3L1	0.686	0.617	0.748	0.575	0.503	0.645
SCr	0.821	0.760	0.871	0.761	0.694	0.819

[a]KDIGO definitions for the diagnosis and staging of AKI, which are based on SCr and UO

Abbreviations: AKI acute kidney injury, AUC-ROC area under the receiver-operating characteristics curve, CI confidence interval, ΔSCr represents $\Delta SCr_{t2\text{-}t0}$, which is the absolute change in SCr between SCr_{t2} and SCr_{t0}, KDIGO Kidney Disease | Improving Global Outcomes, SCHI3L1 serum chitinase 3-like protein 1, SCr serum creatinine, t0 time after the induction of anaesthesia and before the start of surgery, t1 time of intensive care unit admission, t2 2 hours after intensive care unit admission, UCHI3L1 urinary chitinase 3-like protein 1, UCr urinary creatinine, UO urine output

Table S5D | Biomarker performances at t3 for prediction of acute kidney injury

	AKI stage ≥ 2 within 12 h after t1[a]			AKI stage ≥ 1 within 48 h after t1[a]		
	AUC-ROC	Lower limit of 95 % CI	Upper limit of 95 % CI	AUC-ROC	Lower limit of 95 % CI	Upper limit of 95 % CI
ΔSCr	0.938	0.860	1.000	0.643	0.562	0.724
[SCHI3L1]•[UNGAL]	0.774	0.710	0.830	0.665	0.594	0.731
[SCHI3L1]•[UCHI3L1]	0.758	0.692	0.816	0.684	0.613	0.749
[UCHI3L1]•[UNGAL]	0.678	0.610	0.741	0.633	0.563	0.700
[SCr]•[SCHI3L1]	0.814	0.754	0.866	0.723	0.654	0.785
[SCr]•[UNGAL]	0.728	0.660	0.788	0.673	0.602	0.738
[SCr]•[UCHI3L1]	0.754	0.688	0.812	0.725	0.656	0.786
SCHI3L1	0.720	0.653	0.781	0.664	0.593	0.730
UNGAL/UCr	0.649	0.579	0.713	0.600	0.529	0.669
UNGAL	0.656	0.587	0.720	0.612	0.541	0.679
UCHI3L1/UCr	0.653	0.584	0.718	0.617	0.546	0.685
UCHI3L1	0.678	0.610	0.741	0.649	0.578	0.715
SCr	0.857	0.801	0.902	0.792	0.728	0.847

[a]KDIGO definitions for the diagnosis and staging of AKI, which are based on SCr and UO

Abbreviations: AKI acute kidney injury, AUC-ROC area under the receiver-operating characteristics curve, CI confidence interval, ΔSCr represents $\Delta SCr_{t3\text{-}t0}$, which is the absolute change in SCr between SCr_{t3} and SCr_{t0}, KDIGO Kidney Disease | Improving Global Outcomes, SCHI3L1 serum chitinase 3-like protein 1, SCr serum creatinine, t0 time after the induction of anaesthesia and before the start of surgery, t1 time of intensive care unit admission, t3 4 hours after intensive care unit admission, UCHI3L1 urinary chitinase 3-like protein 1, UCr urinary creatinine, UNGAL urinary neutrophil gelatinase-associated lipocalin, UO urine output

SUPPLEMENTAL FIGURES

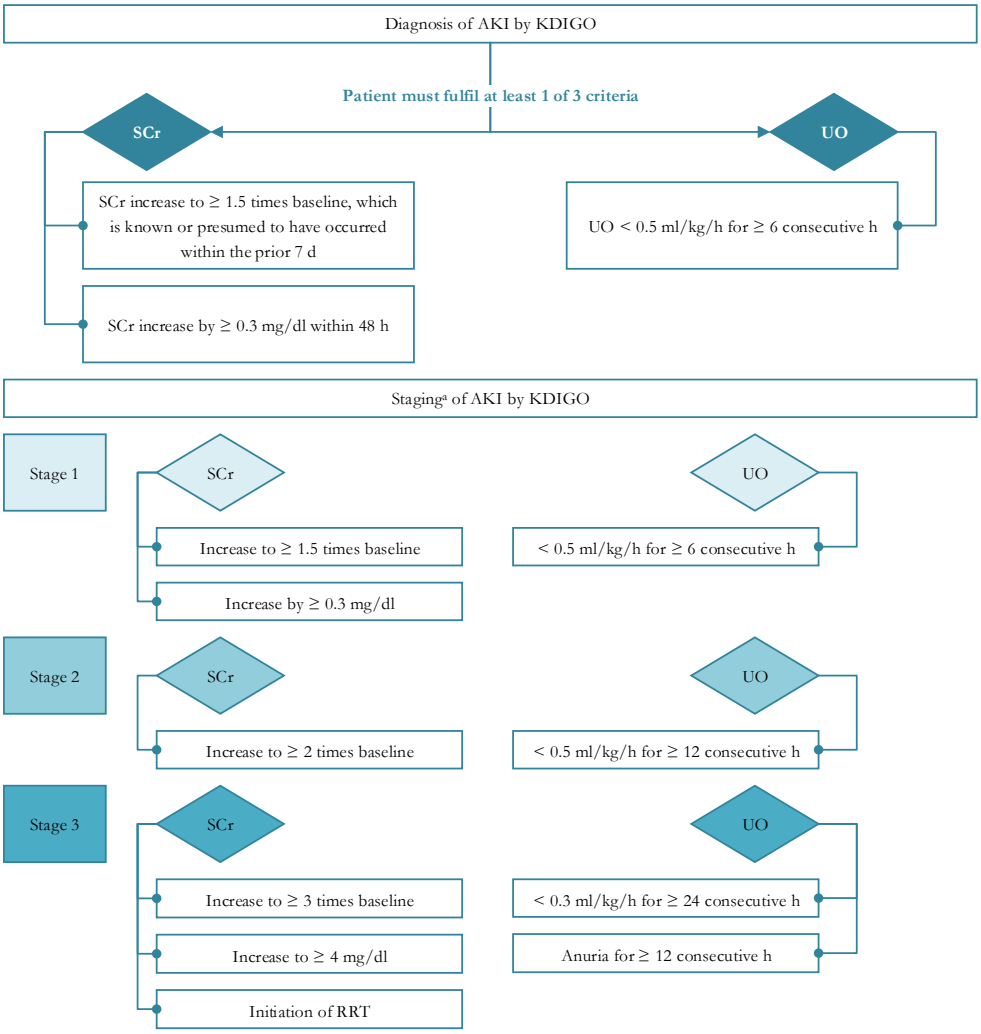


Figure S1 | KDIGO definition and classification of acute kidney injury [11]

[a]For staging purposes, patients should be staged according to the criterion or criteria that give(s) them the highest stage.

Abbreviations: AKI acute kidney injury, KDIGO Kidney Disease | Improving Global Outcomes, RRT renal replacement therapy, SCr serum creatinine, UO urine output

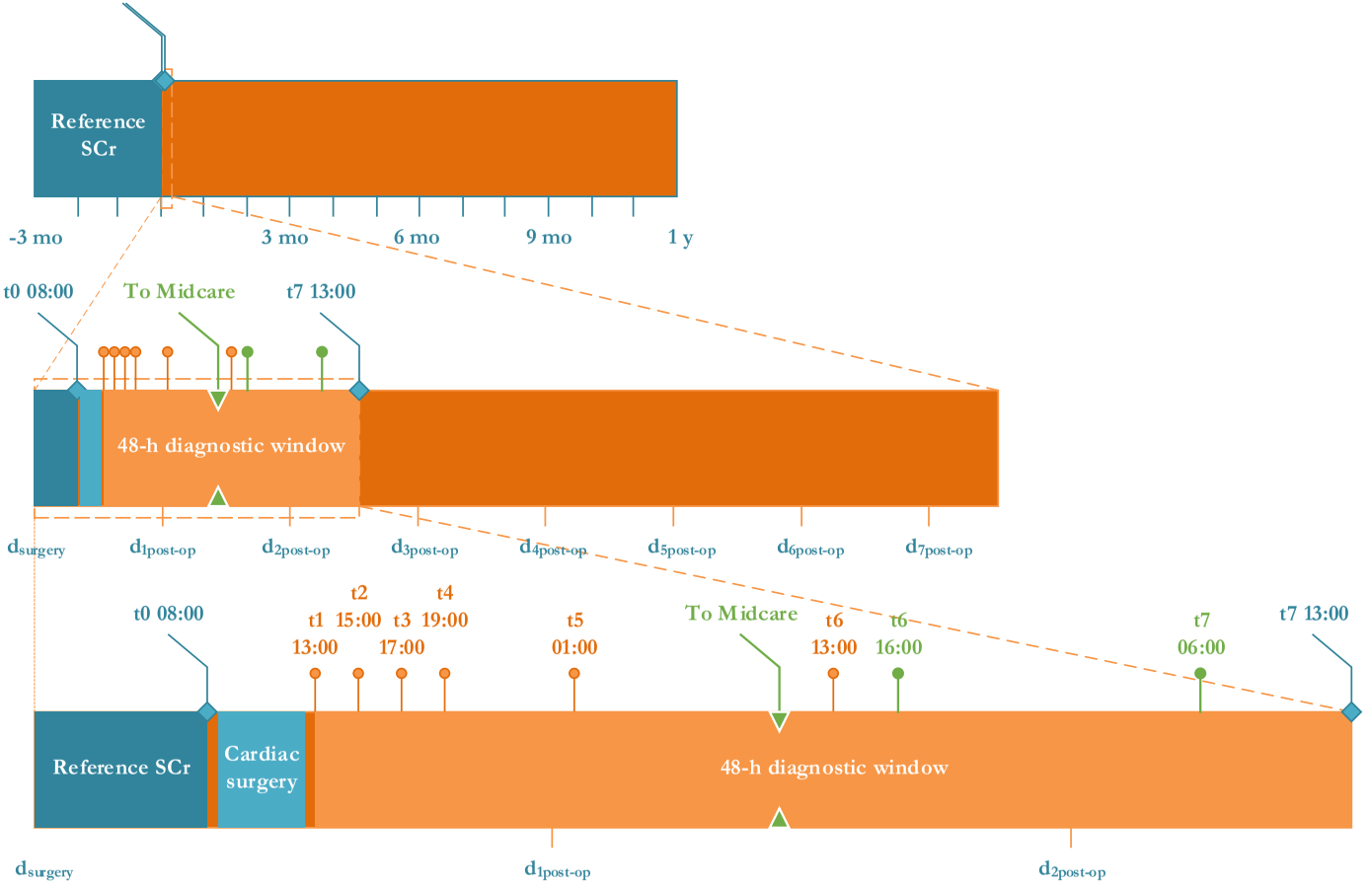


Figure S2A | Study course and sample collection times in a fictional morning patient
Abbreviations: post-op post-operatively, SCr serum creatinine, t time

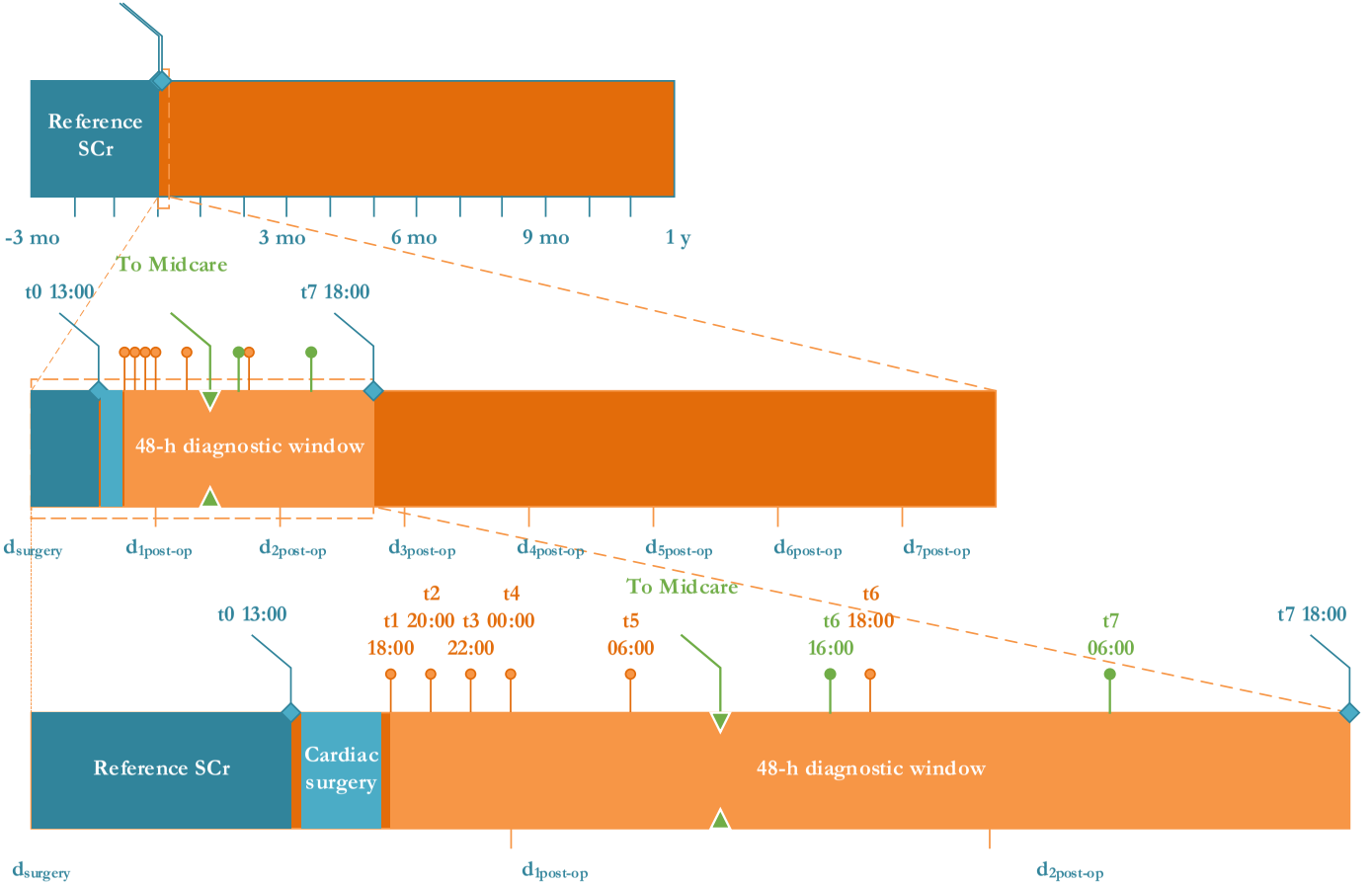


Figure S2B | Study course and sample collection times in a fictional afternoon patient

Abbreviations: post-op post-operatively, SCr serum creatinine, t time

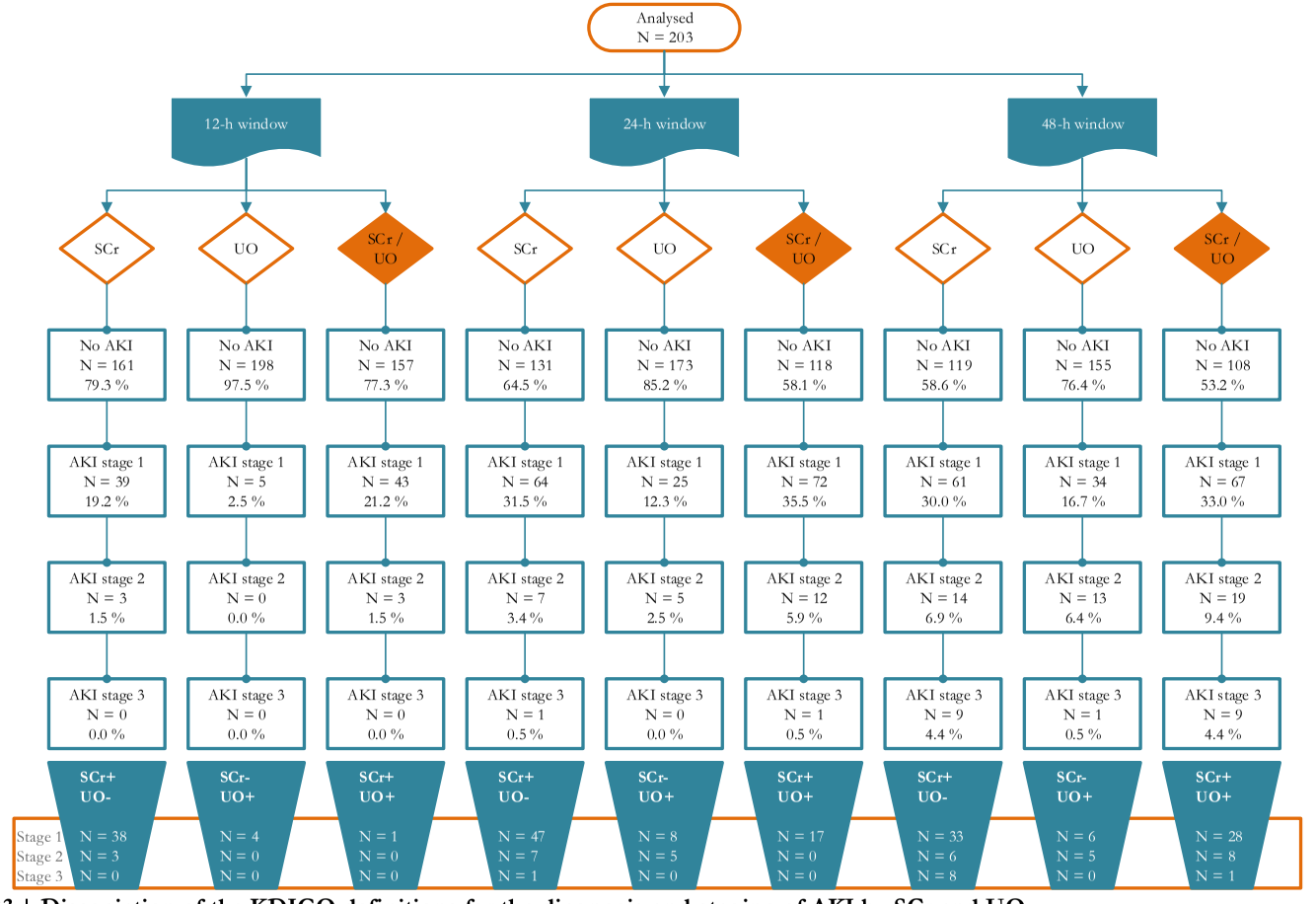


Figure S3 | Dissociation of the KDIGO definitions for the diagnosis and staging of AKI by SCr and UO

Abbreviations: AKI acute kidney injury, KDIGO Kidney Disease | Improving Global Outcomes, SCr serum creatinine, UO urine output

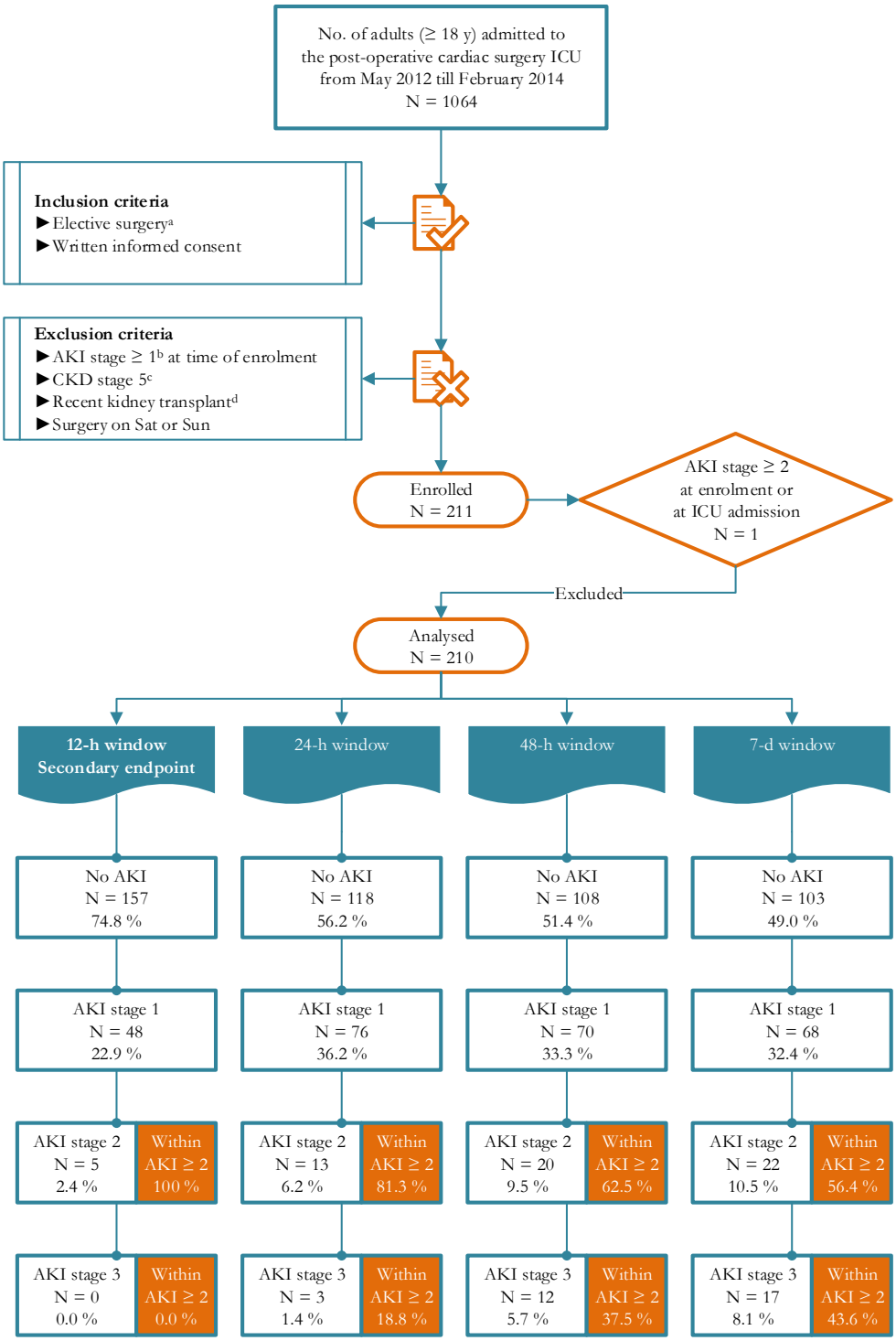

No. of adults (≥ 18 y) admitted to the post-operative cardiac surgery ICU from May 2012 till February 2014
N = 1064
Inclusion criteria
▶Elective surgery[a]
▶Written informed consent
Exclusion criteria
▶AKI stage ≥ 1[b] at time of enrolment
▶CKD stage 5[c]
▶Recent kidney transplant[d]
▶Surgery on Sat or Sun
Enrolled
N = 211
AKI stage ≥ 2 at enrolment or at ICU admission
N = 1
Excluded
Analysed
N = 210
12-h window
Secondary endpoint
24-h window
48-h window
7-d window
No AKI
N = 157
74.8 %
No AKI
N = 118
56.2 %
No AKI
N = 108
51.4 %
No AKI
N = 103
49.0 %
AKI stage 1
N = 48
22.9 %
AKI stage 1
N = 76
36.2 %
AKI stage 1
N = 70
33.3 %
AKI stage 1
N = 68
32.4 %
AKI stage 2
N = 5
2.4 %
Within AKI ≥ 2
100 %
AKI stage 2
N = 13
6.2 %
Within AKI ≥ 2
81.3 %
AKI stage 2
N = 20
9.5 %
Within AKI ≥ 2
62.5 %
AKI stage 2
N = 22
10.5 %
Within AKI ≥ 2
56.4 %
AKI stage 3
N = 0
0.0 %
Within AKI ≥ 2
0.0 %
AKI stage 3
N = 3
1.4 %
Within AKI ≥ 2
18.8 %
AKI stage 3
N = 12
5.7 %
Within AKI ≥ 2
37.5 %
AKI stage 3
N = 17
8.1 %
Within AKI ≥ 2
43.6 %

Figure S4 | Flow of patients over different diagnostic windows for AKI stage ≥ 2

[a]Planned ≥ 4 h in advance

[b]KDIGO definitions for the diagnosis and staging of AKI, which are based on SCr and UO

[c]KDOQI definitions for the diagnosis and staging of CKD

[d]≤ 3 mo before

Abbreviations: AKI acute kidney injury, CKD chronic kidney disease, ICU intensive care unit, KDIGO Kidney Disease | Improving Global Outcomes, KDOQI Kidney Disease Outcomes Quality Initiative, No. number, SCr serum creatinine, UO urine output

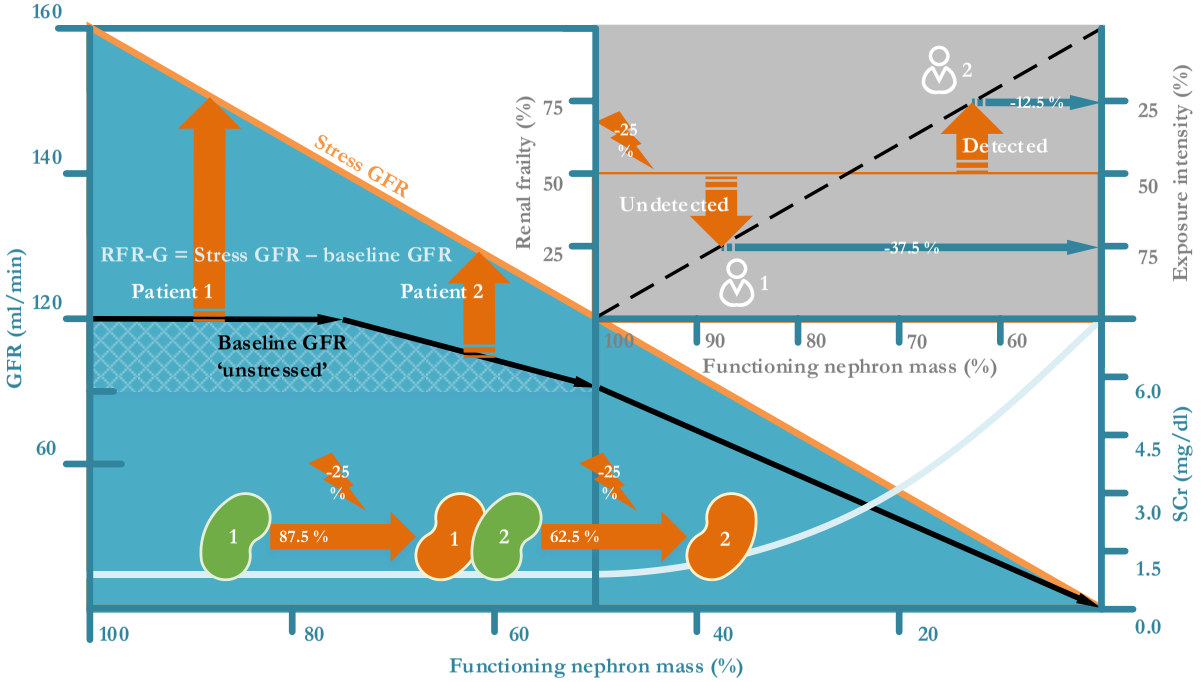


Figure S5 | Renal functional reserve of the glomerular function and functioning nephron mass

Suppose that when all nephrons are functioning baseline GFR is 120 ml/min and that when stressed this GFR can reach 160 ml/min, indicating a RFR-G of 40 ml/min. With 87.5 % functioning nephrons, patient 1 will maintain a baseline GFR of 120 ml/min and reach a lower stress GFR of 150 ml/min. Patient 2 with 62.5 % functioning nephrons will maintain a lower, but still normal, baseline GFR of 105 ml/min and reach a lower stress GFR of 125 ml/min. An exposure that leads to 25 % loss of functioning nephron mass will lead to an increased SCr concentration in patient 2, but will remain undetected in patient 1. Note: the population variability of the RFR-G response is not known; represented values are only illustrative.

Abbreviations: GFR glomerular filtration rate, RFR-G renal functional reserve of the glomerular function

Chapter 5

General Discussion

The scientific aim of this dissertation was to provide clinical validation of the biomarker UCHI3L1 as early diagnostic or 'predictive' tool for emerging AKI in specific adult ICU settings.

I. Main results of this dissertation

In this dissertation we evaluated various biomarkers for early diagnosis or prediction of AKI in two different adult ICU settings. Our adult general ICU setting comprised patients who either underwent non-cardiac surgery or had a non-surgical reason for critical illness, and who had a respiratory SOFA score ≥ 2 or a cardiovascular SOFA score ≥ 1. In this cohort the UCHI3L1 concentration at ICU admission could predict occurrence of AKI stage ≥ 2 within the next 12 h with an AUC-ROC of 0.792 (95 % CI: 0.726-0.849). This performance was similar to that of UNGAL (P = 0.587), which had an AUC-ROC of 0.748 (95 % CI: 0.678-0.810). In our adult elective cardiac surgery ICU setting early post-operative measurements (i.e., within 4 h after ICU admission) of both UCHI3L1 and UNGAL could less satisfactorily predict occurrence of AKI stage ≥ 2 within 12 h after ICU admission. The highest AUC-ROC value for UCHI3L1 was 0.686 (95 % CI: 0.617-0.748) and for UNGAL 0.656 (95 % CI: 0.587-0.720). However, by using the CSA-NGAL score, we found that AKI was predominantly without acute tubular damage in this cohort, making us conclude that renal stress or damage biomarkers were not the most suited biomarkers to measure in these patients. On the other hand, also in this cohort renal stress or damage biomarkers were valuable tools as without UNGAL we would not have been able to identify the patients with subclinical AKI. As alternative, we demonstrated that more frequent assessment of the functional biomarker SCr in the early post-operative ICU period (first 4 h) was the best performing diagnostic tool in these patients. These key findings indicate that SCr assessment should be performed more frequently than in current routine clinical practice in order to detect CSA-AKI more early.

II. A unifying hypothesis explaining the findings in adult cardiac surgery patients

In our adult elective cardiac surgery ICU setting, specific patient characteristics prompted the hypothesis that these patients had a lower RFR-G or a lower functioning nephron mass than the patients in our adult general ICU setting. When the exposure (i.e., the cardiac surgical procedure) caused hypoperfusion with ischemia, it also elicited acute tubular damage as defined by the CSA-NGAL score. We hypothesize that there was a lower risk of subclinical AKI, i.e. diagnostic rise in renal stress or damage biomarkers (e.g., UNGAL) without diagnostic rise in functional biomarkers (e.g., SCr) **(Figure 1, *upper left graph*)**. Further, we hypothesize that in most AKI patients with acute tubular damage, the diagnostic rise in functional biomarkers occurred before the diagnostic rise in renal stress or damage biomarkers due to early failure (i.e., before the development of ischemia) in maintaining a normal whole-organ baseline GFR **(Figure 1, *upper right graph, clinical scenario a-b*)**. Patients with a higher RFR-G or a higher functioning nephron mass may in a timely matter benefit more from measuring renal stress or damage biomarkers, as these biomarkers can then diagnose subclinical AKI **(Figure 1, *upper left graph*)** or predict occurrence of AKI **(Figure 1, *upper right graph, clinical scenario c-d*)**. The lower graphs in Figure 1 also show the correlation between functional biomarkers, renal stress or damage biomarkers, and RFR-G and functioning nephron mass in patients where the exposure caused hypoperfusion without ischemia. Note that in a patient with a normal RFR-G and a normal functioning nephron mass there is a lower probability of reaching the diagnostic threshold of the functional biomarker **(Figure 1, *lower left graph*)**, whereas in a patient with a subnormal RFR-G or a subnormal functioning nephron mass there is a higher probability of reaching the diagnostic threshold of the functional biomarker **(Figure 1, *lower right graph*)**.

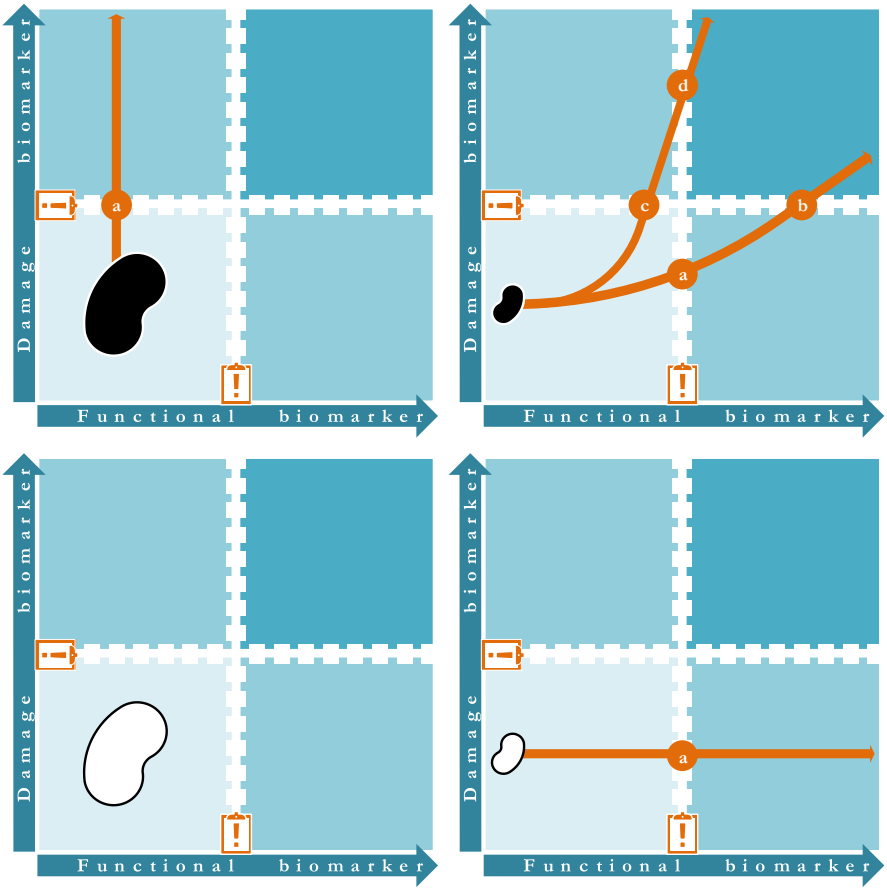


Figure 1 | Correlation between functional biomarkers (e.g., serum creatinine), renal stress or damage biomarkers (e.g., urinary neutrophil gelatinase-associated lipocalin), and renal functional reserve of the glomerular function and functioning nephron mass
The 4 quadrants represent 'no AKI and no acute tubular damage' *(lower left quadrant)*, 'AKI without acute tubular damage' *(lower right quadrant)*, 'subclinical AKI' *(upper left quadrant)* and 'AKI with acute tubular damage' *(upper right quadrant)*.
The letters indicate reaching of a diagnostic threshold. Note that 2 different clinical scenarios are possible in the upper right graph.
Upper graphs: The exposure (i.e., the cardiac surgical procedure) causes hypoperfusion with ischemia (indicated by the black colour of the kidneys)
Lower graphs: The exposure (i.e., the cardiac surgical procedure) causes hypoperfusion without ischemia (indicated by the white colour of the kidneys)
Left graphs: The patient has a normal RFR-G and a normal functioning nephron mass (indicated by the large kidneys)
Right graphs: The patient has a subnormal RFR-G or a subnormal functioning nephron mass (indicated by the small kidneys)
Abbreviations: AKI acute kidney injury, RFR-G renal functional reserve of the glomerular function

III. The long and uncertain path to biomarker utility for AKI diagnosis

A biomarker will not be widely used in the clinic unless all four steps in **Table 1** have been successfully completed [203]. The decision that a biomarker is 'reasonable and necessary' **(Table 1)** indirectly influences coverage by private insurance carriers [204].

Table 1 | Roadmap to biomarker utility in the United States (based on [204])

Step 1 | Biomarker identification			
			File patent application on invention
Step 2 | Biomarker application			
			Describe preferred embodiments of invention
Granting of patent by USPTO		No granting of patent by USPTO	
		No use of biomarker for patient care	
Step 3 | Analytical and clinical validation			
	Document, through use of specific laboratory investigations, that analytical performance characteristics of IVD method are suitable and reliable	Apply manufacturing controls that are adequate to ensure consistent manufacturing of finished IVD product	Document, by conducting clinical trials, that clinical performance characteristics of IVD product are suitable and reliable
FDA approval: 'safe and effective'	No FDA approval		
	No use of biomarker for patient care		
Step 4 | Cost-benefit analysis			
			Conduct RCT
CMS-approval: 'reasonable and necessary'	No CMS-approval		
Routine use of biomarker for patient care	Use of biomarker for patient care		

Abbreviations: CMS Center for Medicare and Medicaid Services, FDA Food and Drug Administration, IVD In Vitro Diagnostic, RCT Randomized Controlled Trial, USPTO United States Patent and Trademark Office

On the roadmap to biomarker utility for AKI diagnosis major hurdles are to be foreseen [205], which are briefly discussed in the following paragraphs.

KDIGO AS THE 'BRONZE STANDARD'

It is of critical importance to understand that we do not have a true 'gold standard' for diagnosis of AKI when validating old (e.g., SCr) and novel biomarkers (e.g., UCHI3L1). Kidney biopsy information would seem a logical tool that may serve as such, however, it is important to realize that nearly all pathologists recognize that overt renal pathology is limited, only making a diagnosis of AKI in clinical context (i.e., basically intact renal morphology, compatible with AKI) [206]. Morphology complemented by immunostaining may enable the identification of an injury pattern that is apparently limited, or obscured by reparative processes [206]. Unlike a 'gold standard', a 'bronze standard' can distort the apparent diagnostic performance of a novel biomarker [207], as illustrated in **Table 2**. This is related to the fact that renal stress or damage may not always be reflected by a decline in GFR (these are the false negatives using KDIGO (n = 6, **Table 2**), assuming that the novel biomarker is in fact 100 % sensitive and specific for AKI), and vice versa, that a decline in GFR may not always reflect renal stress or damage (these are the false positives using KDIGO (n = 8, **Table 2**), assuming that the novel biomarker is in fact 100 % sensitive and specific for AKI). Based on these calculations, it is indicated to use a 'bronze standard' that minimizes false positive misclassifications of disease status, particularly in clinical settings with relatively low expected incidence of true AKI. An appropriate AKI definition may be KDIGO stage ≥ 2 [207]. This AKI endpoint is less prone to prerenal azotaemia and fluctuations in baseline (or 'reference') SCr. Consistent with this suggestion, the multinational cross-sectional AKI-EPI study (n = 1,802) reported that KDIGO stages 2 (OR: 2.945; 95 % CI: 1.382-6.276; P = 0.005) and 3 (OR: 6.884; 95 % CI: 3.876-12.228; P < 0.001), but not KDIGO stage 1 (OR: 1.679; 95 % CI: 0.890-3.169; P = 0.109), were still associated with increased in-hospital mortality in adult ICU patients when adjusted for other variables that may explain mortality [36].

Table 2 | The effect of a 'bronze standard' on the sensitivity and specificity of a novel biomarker that is in fact 100 % sensitive and specific for AKI (based on [207])

	True AKI		
	AKI	No AKI	Total
AKI according to KDIGO	14	8	22
No AKI according to KDIGO	6	72	78
Total	20	80	100
	Sensitivity = 70 %	Specificity = 90 %	

	AKI according to KDIGO		
	AKI	No AKI	Total
Novel biomarker positive	14	6	20
Novel biomarker negative	8	72	80
Total	22	78	100
	Apparent sensitivity = 64 %	Apparent specificity = 92 %	

Abbreviations: AKI acute kidney injury, KDIGO Kidney Disease | Improving Global Outcomes

SUPPORT OF NOVEL BIOMARKERS TO KDIGO

Thanks to de Geus et al., from 2016 onwards acute tubular damage can be staged for severity according to defined UNGAL or plasma NGAL criteria in the cardiac surgery patient [195]. Additionally, novel biomarkers may also support KDIGO staging of AKI, correlating severity stages of renal stress or damage to severity stages of decreased renal function **(Figure 2)** [133]. In 2013 ADQI concluded that there were insufficient renal stress or damage biomarker data to support KDIGO classification and thus also KDIGO definition of AKI **(Figure 2)**. Today, we can state that NGAL already provides additional information to current functional biomarkers in cardiac surgery patients. Indeed, in the absence of diagnostic increases in SCr, NGAL detected patients with likely subclinical AKI who had an increased risk of adverse outcomes [208]. The reliability on cardiac troponin biomarkers to diagnose acute myocardial infarction in the absence of ST-elevation on electrocardiography in patients with unstable angina gives hope that one day clinicians will – in the appropriate clinical context – rely on renal stress or damage biomarkers to diagnose AKI in the absence of a positive KDIGO criterion [121].

NOVEL BIOMARKER THRESHOLD

It is of critical importance to recognize that the threshold for identifying biomarker elevation of a novel renal stress or damage biomarker may be different in each clinical setting, particularly in the

context of sepsis. Considering UNGAL in patients without AKI, higher 'baseline' concentrations were reported for those with sepsis (median 138 ng/ml; 95 % CI: 39-324) than for those without sepsis (median 12 ng/ml; 95 % CI: 5-33) [209, 210]. Likewise, thresholds for identifying biomarker elevations are likely to be different in patients with CKD (i.e., generally higher) [133, 211], making biomarker thresholds not only setting-dependent, but also population-dependent.

Coming back to specifically septic patients, in analogy with UNGAL [157], a part of the urine component of CHI3L1 will likely originate from the circulating plasma pool. Indeed, a non-AKI-specific increase in UCHI3L1 will likely occur when the transport maximum of CHI3L1 is reached, expressed as the maximum transporting capacity of the megalin receptor also used by NGAL [157]. In patients with systemic inflammatory response syndrome **(SIRS)**, plasma CHI3L1 was associated with disease severity: the concentration increased gradually when moving from SIRS without infection through sepsis, severe sepsis, and septic shock [212]. Assessed by AUC-ROC analysis plasma CHI3L1 did not discriminate between SIRS only and sepsis, suggesting that CHI3L1 is a marker of severity of inflammation rather than bacterial load. We speculate that in patients with severe sepsis or septic shock but without AKI as defined by KDIGO, increased UCHI3L1 may perhaps reflect 'subclinical' AKI and not only increased filtered CHI3L1 from the circulating plasma pool. Compared to NGAL which is increased in urine upon UTI (the rate of catheter-associated UTI in the ICU is approximately 2.50 per 1,000 catheter-days [213]), CHI3L1 may have the benefit of being less prone to this interference. Indeed, while bacterial infection leads to induction and secretion of NGAL by superficial epithelial cells facing the lumen of the bladder [214], and infiltrating neutrophils secrete NGAL in urine as well [214], neutrophil-derived CHI3L1 will probably contribute far less to UCHI3L1 in UTI patients. This hypothesis is based on the fact that the secondary granules in one neutrophil contain 0.16 pg CHI3L1 compared to 12 pg NGAL [161, 162]. Nevertheless, whether bacterial infection also leads to induction and secretion of CHI3L1 by the urothelium of the bladder needs to be investigated.

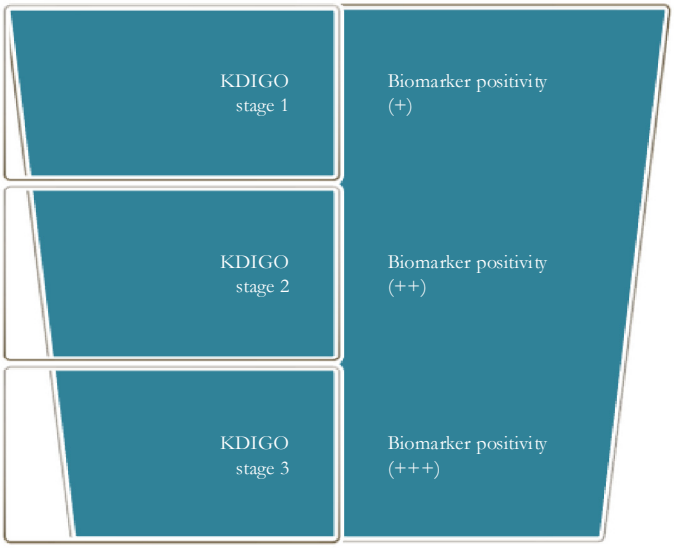


Figure 2 | Proposed novel criteria by the international and interdisciplinary organization Acute Dialysis Quality Initiative for diagnosis and staging of acute kidney injury using functional and renal stress or damage biomarkers (based on [133])
Abbreviations: KDIGO Kidney Disease | Improving Global Outcomes

AETIOLOGY OF AKI

Specific renal biomarkers are linked to specific segments in the nephron as are the site-specific actions of nephrotoxicants **(Table 3)**. Consequently, monitoring of kidney injury molecule-1 (Kim-1, a proximal tubule-specific urinary biomarker) in a patient treated with amphotericin B, which elicits distal tubule-specific toxicity, is not the best option **(Table 3)**. The unique anatomical fingerprint of a renal biomarker thus determines its utility for AKI diagnosis in nephrotoxic AKI [83]. In line with this, it is likely that the utility for AKI diagnosis of novel renal stress or damage biomarkers will be aetiology-dependent in non-nephrotoxic AKI as well. Additionally, novel renal stress or damage biomarkers also have unique (patho)physiological fingerprints, on which we expounded in sections ***III.B.2.ii.*** *"Kidney iron transport: role of neutrophil gelatinase-associated lipocalin"* and ***III.B.3.iii.*** *"Inflammation signalling cascades: role of chitinase 3-like protein 1"* of ***Chapter 1***.

.

Table 3 | The utility of urinary biomarkers to detect injury to specific nephron segments affected by various nephrotoxicants (based on [83])

Glomerulus		Proximal tubules		Loop of Henle		Distal tubules		Collecting duct	
Nephron segment-specific biomarkers of kidney injury	Drugs that elicit site-specific toxicity in the kidney	Nephron segment-specific biomarkers of kidney injury	Drugs that elicit site-specific toxicity in the kidney	Nephron segment-specific biomarkers of kidney injury	Drugs that elicit site-specific toxicity in the kidney	Nephron segment-specific biomarkers of kidney injury	Drugs that elicit site-specific toxicity in the kidney	Nephron segment-specific biomarkers of kidney injury	Drugs that elicit site-specific toxicity in the kidney
Total protein	Doxorubicin	Kim-1	Cyclosporine	Osteopontin	Analgesics (chronic)	Osteopontin	Cyclosporine	Calbindin D28	Amphotericin B
Cystatin C (urinary)	(Adriamycin)	Clusterin	Tacrolimus	NHE-3		Clusterin	Tacrolimus		Acyclovir
β2-microglobulin	Puromycin	NGAL	Cisplatin			GST-μ/π	Sulfadiazine		Lithium (acute)
α1-microglobulin	Gold	GST-α	Vancomycin			NGAL	Lithium (chronic)		
Albumin	Pamidronate	β2-microglobulin	Gentamicin			H-FABP	Amphotericin B		
	Penicillamine	α1-microglobulin	Neomycin			Calbindin D28			
		NAG	Tobramycin						
		Osteopontin	Amikacin						
		Cystatin C (urinary)	Ibandronate						
		Netrin-1	Zoledronate						
		RBP	Hydroxyethyl starch						
		IL-18	Contrast agents						
		HGF	Foscarnet						
		Cyr61	Cidofovir						
		NHE-3	Adefovir						
		Exosomal fetuin-A	Tenofovir						
		L-FABP	Intravenous immune globulin						
		Albumin							

Abbreviations: Cyr cysteine-rich protein, GST glutathione S-transferase, H-FABP heart-type fatty acid-binding protein, HGF hepatocyte growth factor, IL-18 interleukin-18, Kim-1 kidney injury molecule-1, L-FABP liver-type fatty acid-binding protein, NAG N-acetyl-β-glucosaminidase, NGAL neutrophil gelatinase-associated lipocalin, NHE-3 sodium/hydrogen exchanger isoform 3, RBP retinol-binding protein

NOVEL BIOMARKER CONCENTRATION-TIME-COURSE

It is of critical importance to realize that the concentration-time-course of a novel biomarker after stress or damage to the kidneys is unique. This is illustrated in **Figure 3**, where a decline in urinary alkaline phosphatase concentrations with time suggests that this pre-formed brush border enzyme is excreted in diminishing amounts after renal stress or damage [215], whereas a rise in UNGAL concentrations with time is consistent with induction of the this protein after renal stress or damage [215]. This highlights the likely 'window of opportunity' during which a particular biomarker could be used for triaging. The first early intervention study to test the concept that a renal stress or damage biomarker can triage patients at high risk of AKI to early treatment was the EARLYARF trial [216]. In this study the intervention was triggered by a positive reading of the biomarker panel [γ-glutamyltranspeptidase]•[alkaline phosphatase]. Unfortunately, the clinical test performance of this biomarker panel for prediction of emerging AKI was inadequate. The authors had to conclude that imperfect triaging into the intervention arm limited their conclusions regarding the efficacy of early intervention with erythropoietin-β versus placebo

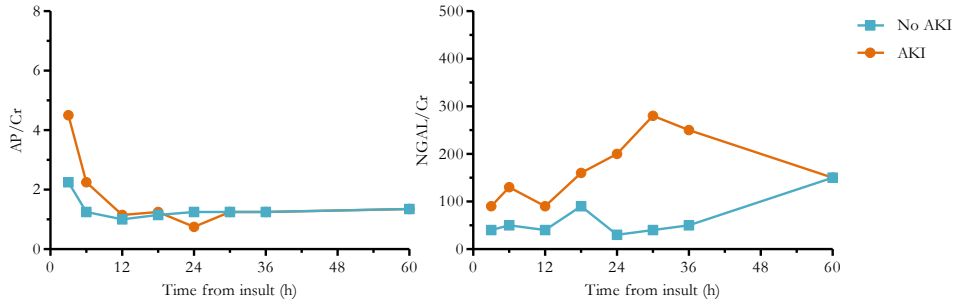


Figure 3 | Concentration-time-course of urinary biomarkers from putative renal insult (based on [216]; created with GraphPad)
Urinary AP concentration in Units/l; urinary NGAL concentration in ng/ml; urinary Cr concentration in mmol/l
Values shown are the mean. There are ±50 measures at each time point for the AKI group.
Abbreviations: AKI acute kidney injury, AP alkaline phosphatase, Cr creatinine, h hour, NGAL neutrophil gelatinase-associated lipocalin

LAB-BASED AUTO-ANALYSER AND BED-SIDE/POINT-OF-CARE BENCH-TOP/TABLE-TOP ANALYSER FORMATS

Expansion of the diagnostic criteria for AKI mandates early reporting of laboratory test results (e.g., novel biomarker concentration), implying the availability of rapid assay methods. Moreover, these assay methods must be available in non-labour-intensive formats (e.g., lab-based auto-analyser or bed-side/point-of-care bench-top/table-top analyser formats). Comparison of measured biomarker concentrations across these formats is important.

In contrast to the traditional centralization of diagnostic analysis in the hospital laboratory, i.e. central laboratory **(CL)** testing, there has been a recent trend towards a more decentralized diagnostic analysis, i.e. point-of-care testing **(POCT)**), which occurs directly at patients' beds – as indicated by "bed-side" –and purports to reduce the turnaround time for test results to reach the clinician by eliminating some of the pre-analytical (e.g., sample transport) and post-analytical (e.g., forwarding of test results) steps [217]. In their systematic narrative review Quinn et al. identified 4 distinct categories of barriers to the clinical adoption of POCT devices [218], which are listed in **Table 4**.

Table 4 | Barriers to hospital-based clinical adoption of point-of-care testing (based on [218])

Barrier 1 | Economic issues
The cost per test of POCT is higher than that of traditional CL testing
The cost-effectiveness of a POCT system is difficult to gauge and cost comparison studies against traditional CL testing methods are complex
The initial costs of implementing a POCT system can be high
The allocation of budgets for POCT is not appropriate
Reimbursement is a major hurdle to POCT implementation
Barrier 2 | Quality assurance and regulatory issues
Device operation by untrained or non-competent staff
Product qualification
Complex regulatory requirements for accreditation
Barrier 3 | Device performance and data management issues
Reduced analytical performance in comparison to centralized laboratory testing
Connectivity and data management problems
Poor usability of devices
Barrier 4 | Staff and operational issues
Reduced levels of staff satisfaction and increased friction between staff groups
Alterations to clinical care pathways
Resistance of the CL to pass control of testing to others
Inappropriate use of POCT
Management structure and clinical governance
Reluctance to change health service practice

The most significant barriers identified are indicated with a lightning bolt.
Abbreviations: CL central laboratory, POCT point-of-care testing

NOVEL BIOMARKER COST-EFFECTIVENESS

Last but not least, the cost-effectiveness ratio of validated novel biomarkers must be investigated in order to convince policymakers to redistribute financial resources towards use of these markers in the clinic. At present, it is still uncertain whether early diagnosis of AKI by a novel more expensive biomarker is cost-effective. Using a decision analysis model for a base case (i.e., a 67-year-old male in the United Kingdom undergoing CABG surgery without a history of CKD admitted to the ICU immediately after the procedure), Shaw et al. calculated a cost-effectiveness ratio of £358 per quality-adjusted life-year for the NGAL strategy (i.e., the use of UNGAL in addition to current clinical practice for the diagnosis of AKI) compared with £396 per quality-

adjusted life-year for the standard approach (i.e., monitoring of SCr, blood urea nitrogen and UO) [219]. A quality-adjusted life-year takes into account longevity and quality-of-life [220]. The number of quality-adjusted life-years is estimated by multiplying the years of survival by quality-of-life measured on a scale from zero (equivalent to death) to 1 (perfect health) [220]. Recently, Meersch et al. showed that the use of an AKI care bundle (i.e., intervention therapy) vs. a standard care bundle (i.e., control therapy) targeted at a cohort of adult cardiac surgery patients at high risk for AKI could reduce the occurrence and severity of AKI [26]. The risk for AKI was determined by the low 0.3 cutoff of the NephroCheck® test. This threshold has a very high negative predictive value (i.e., 97 %) meaning that patients testing positive are at much higher risk for AKI than those testing negative. Göcze et al. used the same 0.3 cutoff for risk stratification in adult ICU patients after major elective non-cardiac surgery and could demonstrate a reduced occurrence and severity of AKI in the intervention group (AKI care bundle) compared to the control group (standard care bundle) in a subgroup (i.e., NephroCheck® test result ≤ 2.0) analysis [27]. These authors did not calculate the cost-effectiveness ratio for this intervention, but given the fact that Chertow et al. found that even SCr increases by ≥ 0.3 mg/dl in hospitalized adults are associated with a 4.1-fold increase (95 % CI: 3.1-5.5) in the odds of in-hospital death and US$4,886 in excess hospital costs [221], the cost-effectiveness ratio of the use of an AKI care bundle in adult cardiac surgery patients at high risk for AKI (as identified by the NephroCheck® test) may indeed be lower than that of the use of a standard care bundle.

POSITIONING OF UCHI3L1, UNGAL AND URINARY [TIMP-2]•[IGFBP7] TOWARDS THESE HURDLES

A cautious estimate on how far the biomarkers UCHI3L1, UNGAL and urinary [TIMP-2]•[IGFBP7] have come today on the roadmap to biomarker utility for AKI diagnosis is provided in **Table 5**. None of these three biomarkers could evade the use of KDIGO as the 'bronze standard' test in the evaluation of their diagnostic test performance for AKI.

Table 5 | Positioning of UCHI3L1, UNGAL and urinary [TIMP-2]•[IGFBP7] towards hurdles on the roadmap to biomarker utility for AKI diagnosis

Hurdle	UCHI3L1	UNGAL	Urinary [TIMP-2]•[IGFBP7]
Support to KDIGO	**X**	**Preliminary**: CSA-NGAL score [195]	**Preliminary**: RCTs with risk stratification based on 0.3 cutoff [26, 27]
		Adult cardiac surgery	Adult cardiac surgery Adult major elective non-cardiac surgery
Threshold	**Preliminary**: Chapter 3 [193]	CSA-NGAL score [195]	Opal study [140]
		Stage 1 cutoff Considered to have high NPV Stage 2 cutoff Considered to have high PPV	0.3 cutoff NPV (%) = 97 (95 % CI: 96-99) 2.0 cutoff PPV (%) = 49 (95 % CI: 41-58)
	Adult non-cardiac surgery or non-surgical reason for critical illness **(need for multicentre study)**	Adult cardiac surgery	Adult non-cardiac surgery or non-surgical reason for critical illness
Aetiology of AKI	Background: major source of biomarker in AKI		
	Intrarenal macrophages upon activation in the renal milieu [110]	Cells of distal tubule origin [89]	TIMP-2: cells of distal tubule origin [222] IGFBP7: cells of proximal tubule origin [222]
	Preliminary: Chapter 3 [193]	Sapphire study [136] Inferior to [TIMP-2]•[IGFBP7]	Sapphire study [136] Superior to UNGAL AUC-ROC = 0.80 (95 % CI: 0.74-0.84)
	Adult non-cardiac surgery or non-surgical reason for critical illness Presumed: poor specificity in sepsis as two "marker-sensitive" conditions overlap in one patient [212]	Adult non-cardiac surgery or non-surgical reason for critical illness Poor specificity in sepsis as two "marker-sensitive" conditions overlap in one patient [223]	Adult non-cardiac surgery or non-surgical reason for critical illness Sapphire and Topaz sub-analysis: test performance is not affected by non-renal organ dysfunction [224]
		TRIBE-AKI consortium AUC-ROC = 0.67 (SE: 0.04) [168]	
	Need for evaluation in adult cardiac surgery cohort with evidence of acute tubular damage according to CSA-NGAL score (cf. Chapter 4, [225])	Adult cardiac surgery	**Need for multicentre study** in adult cardiac surgery
Concentration-time-course	**X**	Within 6 h after insult [135]	**X**
		Adult cardiac surgery	
Analyser format	**X**	Lab-based auto-analyser format (BioPorto, NGAL® test)	Bed-side/point-of-care bench-top/table-top analyser format (Astute Medical, NephroCheck® test)
	ELISA (e.g., R&D Systems, Quantikine®		

	ELISA Human Chitinase 3-like 1 Immunoassay)		
Cost-effectiveness	Cost per test		
	€ 6-10 (RUO)	€ 30-50 (IVD)	€ 30-50 (IVD)
	X	X	**Preliminary**: RCTs with risk stratification based on 0.3 cutoff [26, 27]

Abbreviations: AKI acute kidney injury, AUC-ROC area under the receiver-operating characteristics curve, CI confidence interval, CSA cardiac surgery-associated, IGFBP7 insulin-like growth factor-binding protein 7, IVD In Vitro Diagnostic, NPV negative predictive value, PPV positive predictive value, RUO Research Use Only, TAL thick ascending limb of loop of Henle, TIMP-2 tissue inhibitor of metalloproteinases-2, UCHI3L1 urinary chitinase 3-like protein 1, UNGAL urinary neutrophil-gelatinase associated lipocalin

IV. Neutrophil gelatinase-associated lipocalin for canine AKI diagnosis

Zhou et al. randomized purpose-bred research beagle dogs to either daily intramuscular injection with saline for 9 d or daily intramuscular injection with gentamicin for 9 d [226]. AUC-ROC curves of different biomarkers at d3, d6 or d9 were plotted for the outcome 'histopathological changes of kidneys' (i.e., tubular cell necrosis or tubular cell hyaline droplet formation) at d3, d6 or d9 (no lesions were observed in control dogs). This analysis (including all time points) revealed that UNGAL/UCr was superior to SCr for the detection of acute tubular damage (AUC-ROC of 1.000 (95 % CI: 0.903-1.000) for UNGAL/UCr versus 0.872 (95 % CI: 0.718-0.959) for SCr; P = 0.029).

In another study biomarker concentrations after acute haemorrhage (via femoral artery catheter) and colloid-mediated reperfusion in anaesthetized Greyhound dogs were measured [227]. Histology confirmed that the model successfully induced acute tubular damage. Increases in UNGAL and UNGAL/UCr from baseline were observed as early as 3 h following the inciting cause ($P < 0.050$). An increase in SCr (IRIS grade 2) was evident immediately following hypotension, suggesting that this resulted from a pre-renal functional change. Despite the observation that AKI was not purely pre-renal, SCr did not increase further during reperfusion. This may be explained by volume resuscitation leading to dilution, and so blunting of SCr increase.

The novel biomarker UNGAL has also been investigated in dogs with naturally occurring AKI. The first study to investigate UNGAL prospectively enrolled 39 dogs that were to undergo surgery [228]. Dogs with azotaemia or relevant renal historical/clinical/imaging signs before surgery were excluded. Pre-operative SCr was considered as the baseline. The endpoint was AKI defined as an acute increase in SCr of ≥ 0.3 mg/dl from baseline within 48 h. Biomarkers were measured before surgery and at 12 h, 24 h, 48 h and 72 h after surgery. AKI occurred in 30.8 % of the canine patients. Compared with the no AKI group, UNGAL increased at 12 h post-surgery in dogs with AKI (P = 0.022), while SCr was only increased at 24 h post-surgery (P = 0.040). Another cohort study prospectively enrolled 30 dogs with heatstroke [229]. SCr was measured at presentation, 4 h post presentation and then every 12 h until discharge or death. AKI occurred in 63.3 % of the canine patients (IRIS guidelines implemented after fluid resuscitation; UO was not measured). Compared with the no AKI group, UNGAL/UCr was increased at presentation in dogs with AKI (P = 0.006). Although measured GFR (Cr clearance) after fluid resuscitation (4 h post presentation) was < 1 ml/min/kg in 69.2 % of dogs (normal GFR in dog is > 2 ml/min/kg), 50 % of dogs were non- or only mildly azotaemic at that time, emphasizing the insensitivity of SCr.

Overall, the results from these 4 studies illustrate the limitations of SCr and the potential of the acute tubular damage biomarker UNGAL in dogs, and so underline the urgent need for this and other novel renal stress or damage biomarkers, thus corroborating the findings in human patients.

In the context of sepsis, it is presumed that UNGAL has a poor specificity for AKI in septic dogs as two "marker-sensitive" conditions overlap in these patients. Both serum NGAL and UNGAL/UCr at admission were higher in dogs presented with no AKI and sepsis requiring emergency laparotomy (n= 15) than in dogs presented with no AKI and intervertebral disk disease requiring emergency surgical intervention (n = 10) [230]. An alternative explanation is

that similar to humans, sepsis is associated with renal stress or damage and subclinical AKI, leading to increased NGAL. In dogs with negative urine culture (n = 33), the severity of pyuria correlated with the concentration of UNGAL [231], suggesting that in dogs with UTI [232], UNGAL is influenced not only by bacteriuria (induction and secretion of NGAL by superficial epithelial cells facing the lumen of the bladder [214]), but also by pyuria.

In addition to the use of NGAL as a biomarker for AKI in dogs, Bland et al. demonstrated kidney injury molecule-1 (KIM-1) expression in the feline kidney and identified its shedding into urine of cats at risk for AKI [233]. Performance data are lacking to date, but based on these preliminary findings, further investigation of KIM-1 as a biomarker of AKI in cats appears warranted.

At present, there are to the best of our knowledge no published data on the use of UCHI3L1 and urinary [TIMP-2]•[IGFBP7] as biomarkers for AKI in companion animals.

V. Biomarkers for AKI management

Although this dissertation focused on diagnostic AKI biomarkers, it should be emphasized that the context of use of biomarkers is much broader (cf. preferred embodiments of an invention as stated in ***Chapter 2***) **(Figure 4)**. Also, it should be noted that causal and sensitizing AKI risk factors, or exposures and susceptibilities, form the pillars of AKI risk assessment **(Figure 4)** [11].

Our invention (filed as international patent application WO2012/136548) has other preferred embodiments than 'a method wherein the correlation step comprises assigning a diagnosis of the occurrence or non-occurrence of AKI to the subject based on the assay result'. Another preferred embodiment is 'a method wherein the correlation step comprises assessing whether or not renal function is improving or worsening in a subject who has suffered from AKI based on the assay result'. In accordance with this, Schmidt et al. independently showed that UCHI3L1 predicts the occurrence of delayed graft function in adult patients who receive deceased-donor kidney transplants [110]. Recently, the same group also studied a cohort of hospitalized patients

with AKI and found that UCHI3L1 was associated with the composite outcome of AKI progression and in-hospital death [156].

Recently, Montgomery et al. used unilateral renal IRI as a robust model for acute to chronic kidney injury in mice [234, 235]. Based on their findings, the authors proposed a model of CHI3L1-mediated macrophage-myofibroblast crosstalk during kidney fibrosis **(Figure 5)** [234], suggesting that translational investigation of CHI3L1 as an outcome AKI biomarker (i.e., progression to AKD or CKD) in humans is warranted.

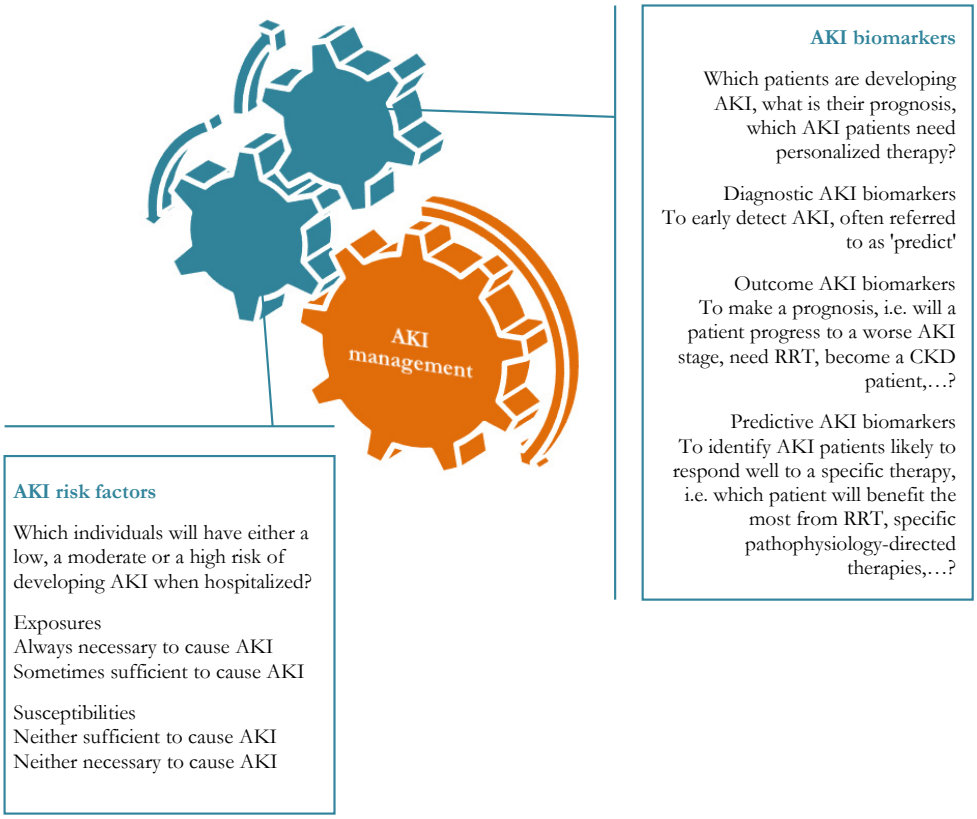


Figure 4 | The gear wheels 'AKI risk factors' and 'AKI biomarkers' set into motion a good AKI management (based on [121])
Only when both these gear wheels work well, the 'AKI management' gear wheel will turn around smoothly. AKI management comprises exposure containment, preventive intervention or therapy, supportive intervention or therapy, and follow-up.
Abbreviations: AKI acute kidney injury, CKD chronic kidney disease, RRT renal replacement therapy

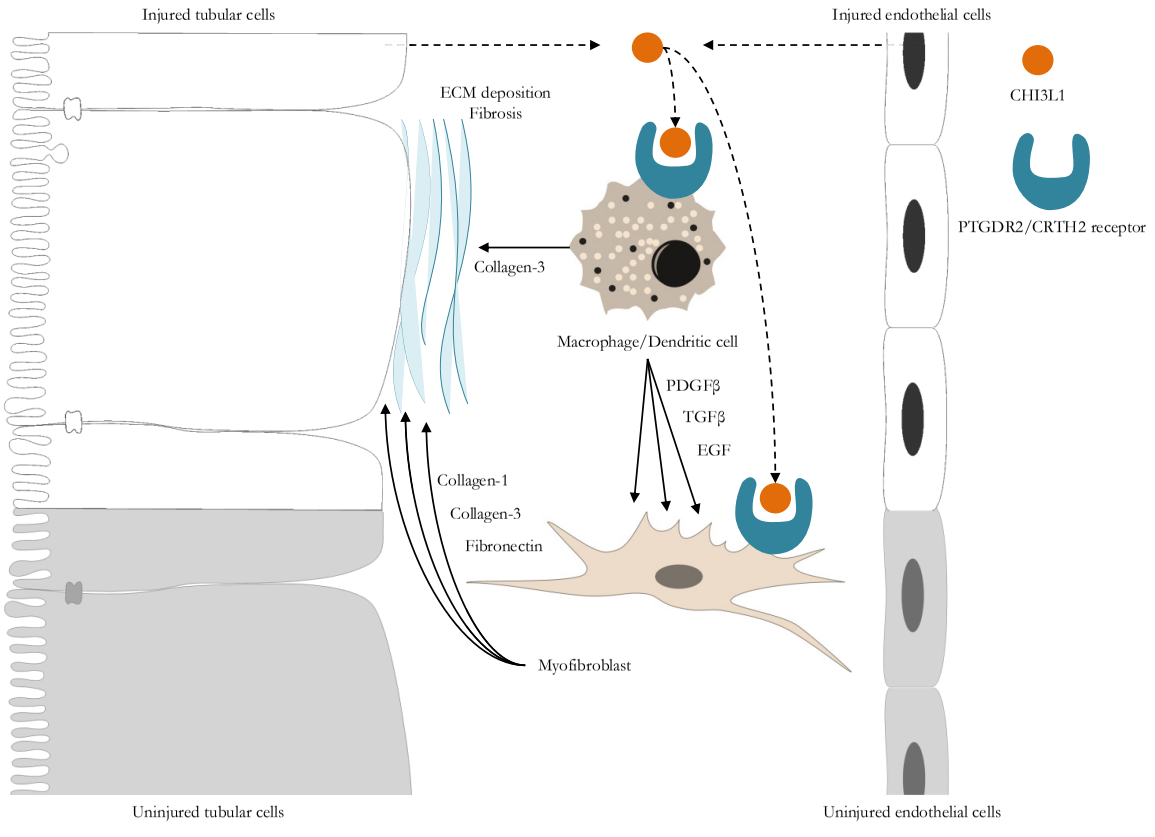


Figure 5 | A proposed model of CHI3L1-mediated macrophage-myofibroblast crosstalk during kidney fibrosis (based on [234]; created with Motifolio)
"Sustained low-level expression of CHI3L1 by non-repaired tubular cells and/or endothelial cells is proposed to activate the PTGDR2/CRTH2 receptor on macrophages, inducing their secretion of both collagen-3 and pro-fibrotic growth factors, such as PDGFβ and TGFβ; this, in turn, can act separately or cooperatively with CHI3L1 to stimulate myofibroblast expression of additional extracellular matrix (ECM) components, including collagen-1 and fibronectin. Dashed lines, proposed signalling; solid lines, analysed signalling."
Abbreviations:
CHI3L1 chitinase 3-like protein 1, EGF epidermal growth factor, PDGFβ platelet-derived growth factor β, PTGDR2/CRTH2 prostaglandin D2 receptor 2/chemokine receptor homologous molecule expressed on Th2 lymphocytes, TGFβ transforming growth factor β

VI. Future prospects

RANDOMISED CONTROLLED TRIAL

The question that should be asked by any clinician is: "in what respect does the result of the biomarker test changes the management and treatment of my patient?". As mentioned, Meersch et al. showed that the use of an AKI care bundle (i.e., intervention therapy) vs. a standard care bundle (i.e., control therapy) targeted at a cohort of adult cardiac surgery patients at high risk for AKI (as identified by the NephroCheck® test) could reduce the occurrence and severity of AKI. A first future prospect is to test whether clinical outcomes (most likely intermediate endpoints such as AKI stage, but ideally hard endpoints such as new requirement for RRT, mortality or new-onset CKD [236]) are improved in patients that do versus do not undergo renal stress or damage biomarker testing with subsequent clinical decision making on the basis of these biomarker results. An example of a randomised controlled trial of a diagnostic test is the RATPAC trial [237], which aimed to evaluate the clinical effectiveness of using a point-of-care cardiac marker panel in patients presenting to the emergency department with suspected but not proven acute myocardial infarction. Participants were allocated to receive either diagnostic assessment using the point-of-care cardiac marker panel or conventional diagnostic assessment without the panel. All tests and treatments other than the panel were provided at the discretion of the clinician. The primary outcome was the proportion of patients successfully discharged home, defined as patients with a discharge decision having been made at 4 h after initial presentation and without any major adverse event during the following 3 mo. The authors concluded that the POCT increased the proportion of patients successfully discharged home. Importantly, when comparing this proportion between the 6 participating hospitals, the clinical effectiveness of using the point-of-care cardiac marker panel varied markedly [238]. This indicates that simple provision of rapid biomarker results will be ineffective unless it is accompanied by treatment decision.

SINGLE BIOMARKER VERSUS BIOMARKER PANEL

A second future prospect is to test whether combining novel renal stress or damage biomarkers to be used either concurrently in a biomarker panel in analogy with the NephroCheck® test [TIMP-2]•[IGFBP7], or one by one as confirmatory evidence, would improve the clinical performance characteristics of both single biomarkers.

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Summary

Acute kidney injury **(AKI)** is an abrupt loss of kidney function. Over the years this syndrome has had many names, like ischuria renalis (1802), war nephritis (1914), crush syndrome (1939) and acute renal failure **(ARF)** (1951). Only with the birth of the 'Risk, Injury, Failure, Loss, and End-stage renal disease' **(RISK)** system in 2004, AKI had its first consensus definition. RISK defined acute changes in kidney function using serum creatinine **(SCr)** and urine output **(UO)** as criterion. Revised systems for the diagnosis and staging of AKI followed, with in 2007 the Acute Kidney Injury Network **(AKIN)** system, and in 2012 the Kidney Disease | Improving Global Outcomes **(KDIGO)** system.

As logical approach to AKI the **FIRST CHAPTER** starts by describing kidney function. Subsequently, the AKI syndrome is expounded with in-depth exploration of the current consensus definition of AKI (including its limitations), the high incidence of AKI and the poor outcomes of AKI. Finally, the latest understanding of the pathophysiology of AKI as logical approach to novel biomarkers for AKI is summarized. Based on this knowledge, experts proposed to name the pre-phase that leads to AKI 'acute kidney stress', which is debatably described as a condition of either renal stress or initial renal damage. Ideally, urinary biomarkers can localize renal stress or damage to a specific nephron site. As regulator of kidney iron transport, urinary neutrophil gelatinase-associated lipocalin **(UNGAL)** is a clinically validated biomarker for the early diagnosis or 'prediction' of emerging AKI in different intensive care unit **(ICU)** settings. This biomarker herewith showed a good clinical test performance in numerous studies.

The **SECOND CHAPTER** states the scientific aim of the two clinical studies that were conducted, which was to provide clinical validation of the novel candidate biomarker urinary chitinase 3-like protein 1 **(UCHI3L1)** as early diagnostic or 'predictive' tool for emerging AKI in specific adult ICU settings. This regulatory protein of signalling in kidney tubular epithelial cells' and

macrophages' inflammation signalling cascades, was discovered in 2012 at the Faculty of Veterinary Medicine of Ghent University by urinary proteomics in a novel mouse model for sepsis-induced AKI.

The **THIRD CHAPTER** describes a single-center (Ghent University Hospital) prospective cohort study in the adult surgical and medical – or 'general' – ICU setting, which aimed to provide clinical validation of the biomarker UCHI3L1 in patients who either underwent non-cardiac surgery or had a non-surgical reason for critical illness. AKI was diagnosed and staged according to the KDIGO system, which is based on SCr and UO. One-hundred eighty-one patients who had either a respiratory Sepsis-related Organ Failure Assessment **(SOFA)** score ≥ 2 or a cardiovascular SOFA score ≥ 1, and did not yet have AKI stage ≥ 2 at time of enrolment, were included. Twenty-one patients (12 %) developed AKI stage ≥ 2 within 7 days **(d)** after enrolment, of which 6 (3 %) within the first 12 hours **(h)**. The concentration of UCHI3L1 at enrolment predicted the occurrence of AKI stage ≥ 2 within the next 12 h, in which the probability that UCHI3L1 correctly ranked a randomly chosen case / non-case pair with respect to their measured concentrations, was high. This test performance and that of the biomarker UNGAL measured at enrolment were of equal value. In-depth study of the biomarker UCHI3L1 further showed that increasing UCHI3L1 concentrations were associated with an increasing severity of AKI. This study allowed to conclude that in the adult 'general' ICU setting UCHI3L1 is a clinically validated biomarker for the early diagnosis or prediction of AKI stage ≥ 2 occurring within 12 h after its measurement. The biomarker UCHI3L1 herewith showed a good clinical test performance.

The **FOURTH CHAPTER** describes a single-centre (Ghent University Hospital) prospective cohort study in the adult elective cardiac surgery ICU setting, which aimed to provide clinical validation of the biomarker UCHI3L1 in patients who underwent elective cardiac surgery. AKI was diagnosed and classified according to the KDIGO system, which is based on SCr and UO. Acute

tubular damage was diagnosed according to the UNGAL score developed for use in adult cardiac surgery patients. The biomarkers UCHI3L1, UNGAL and SCr were measured more frequently than SCr in routine early post-operative ICU practice. Of the 211 enrolled elective cardiac surgery patients, 203 patients who had no AKI pre-operatively and at time of post-operative ICU admission **(t1)** were included in the analysis of the primary endpoint (i.e., AKI stage ≥ 1 within 48 h after t1), while 210 patients without AKI stage ≥ 2 pre-operatively and at t1 were included in the analysis of the secondary endpoint (i.e., AKI stage ≥ 2 within 12 h after t1). Within 48 h after t1, 95 patients (46.8 %) had developed AKI, of which 67 (70.5 %) had stage 1, 19 (20.0 %) stage 2 and 9 (9.5 %) stage 3. AKI was classified predominantly as AKI without acute tubular damage (84.6 %). During the first 4 h postoperatively, only SCr predicted the occurrence of AKI within 48 h after t1, in which the probability that SCr correctly ranked a randomly chosen case / non-case pair with respect to their measured concentrations, was high. In the same period, both UCHI3L1 and UNGAL predicted the occurrence of AKI stage ≥ 2 within 12 h after t1. However, the probability herewith that either UCHI3L1 or UNGAL correctly ranked a randomly chosen case / non-case pair with respect to their measured concentrations, was low. Moreover, this test performance was inferior to both that of SCr and that of the absolute change in SCr from the pre-operative measurement to early post-operative measurements. This study allowed to conclude that in the adult elective cardiac surgery ICU setting UCHI3L1 is a clinically validated biomarker for the early diagnosis or prediction of AKI stage ≥ 2 occurring within 12 h after its measurement. The biomarker UCHI3L1 herewith showed, however, an inadequate clinical test performance. This marked difference with the first clinical validation study could be explained by the finding that AKI was classified predominantly as AKI without acute tubular damage.

In the final **FIFTH CHAPTER** the results of both clinical studies are discussed and major hurdles on the roadmap to biomarker utility for AKI diagnosis as well as future prospects are defined.

Samenvatting

Acute nierinsufficiëntie **(ANI)** staat voor een plots functieverlies van de nier. Dit syndroom kende door de jaren heen verschillende benamingen, zoals renale ischurie (1802), oorlogs-nefritis (1914), crush syndroom (1939) en acuut nierfalen **(ANF)** (1951). De eerste consensus definitie van ANI kwam er pas in 2004 met het 'Risk, Injury, Failure, Loss, and End-stage renal disease' **(RISK)** systeem. RISK definieerde acute veranderingen in de nierfunctie gebruik makend van serum creatinine **(SCr)** en urine debiet **(UD)** als maatstaf. Aangepaste systemen voor de diagnose en stagering van ANI volgden, met in 2007 het 'Acute Kidney Injury Network' **(AKIN)** systeem, en in 2012 het 'Kidney Disease | Improving Global Outcomes' **(KDIGO)** systeem.

Als logische aanloop naar ANI start het **EERSTE HOOFDSTUK** met het beschrijven van de nierfunctie. Vervolgens wordt het ANI syndroom toegelicht met uitdieping van de huidige consensus definitie van ANI (inclusief limitaties), de hoge incidentie van ANI en de slechte uitkomsten van ANI. Tenslotte worden de laatste inzichten in de pathofysiologie van ANI samengevat als logische aanloop naar nieuwe biomerkers voor ANI. Op basis van deze kennis stelden experten voor om de pre-fase die leidt tot ANI te benoemen als 'acute nier-stress'. Dit is een conditie die dubbelzinnig omschreven wordt als hetzij renale stress, hetzij renale schade. Urinaire biomerkers kunnen idealiter renale stress of schade specifiek lokaliseren in het nefron. Als regulator van het ijzertransport in de nier is urinair 'neutrophil gelatinase-associated' lipocaline **(UNGAL)** een klinisch gevalideerde biomerker voor de vroege diagnose of 'predictie' van opkomende ANI in verschillende intensieve zorg afdeling **(IZA)** omgevingen. Deze biomerker liet hierbij een goede klinische testprestatie zien in talrijke studies.

Het **TWEEDE HOOFDSTUK** geeft het wetenschappelijke doel van de twee uitgevoerde klinische studies aan, namelijk het klinisch valideren van de nieuwe kandidaat biomerker urinair 'chitinase 3-like' proteïne 1 **(UCHI3L1)** als vroeg diagnostische of 'voorspellende' test voor opkomende ANI in specifieke adulte IZA omgevingen. Dit regulator-eiwit van inflammatoire signalisatie in

tubulaire cellen van de nier en macrofagen werd in 2012 aan de faculteit Diergeneeskunde van de Universiteit van Gent ontdekt via urinair proteoom onderzoek in een nieuw muismodel voor sepsis-geïnduceerde ANI.

Het **DERDE HOOFDSTUK** beschrijft een unicenter (Universitair Ziekenhuis Gent) prospectieve cohortstudie in de adulte heelkundige en medische – of 'algemene' – IZA omgeving. Deze eerste studie beoogde de klinische validatie van de biomerker UCHI3L1 in patiënten die ofwel niet-cardiale heelkunde ondergingen, ofwel kritisch ziek waren door een andere oorzaak dan heelkunde. De diagnose en stagering van ANI gebeurden volgens het KDIGO systeem dat gebaseerd is op SCr en UD. Honderd eenentachtig patiënten die ofwel een respiratoire 'Sepsis-related Organ Failure Assessment' **(SOFA)** score ≥ 2 hadden, ofwel een cardiovasculaire SOFA score ≥ 1, en nog geen ANI stadium ≥ 2 hadden bij rekrutering, werden geïncludeerd. Eenentwintig patiënten (12 %) ontwikkelden ANI stadium ≥ 2 binnen de 7 dagen **(d)** na rekrutering, waarvan 6 (3 %) binnen de eerste 12 uur **(u)**. De concentratie van UCHI3L1 bij rekrutering voorspelde het optreden van ANI stadium ≥ 2 in de eerstvolgende 12 u, waarbij de kans dat UCHI3L1 een willekeurig gekozen ziek / niet-ziek paar correct rangschikte gebaseerd op hun gemeten concentraties, hoog was. Deze testprestatie was evenwaardig aan die van de biomerker UNGAL gemeten bij rekrutering. Grondige studie van de biomerker UCHI3L1 toonde verder dat stijgende concentraties geassocieerd waren met een stijgende ernst van ANI. Deze studie liet toe te besluiten dat in de adulte 'algemene' IZA omgeving UCHI3L1 een klinisch gevalideerde biomerker is voor de vroege diagnose of predictie van ANI stadium ≥ 2 optredend binnen de 12 u na zijn meting. De biomerker UCHI3L1 liet hierbij een goede klinische testprestatie zien.

Het **VIERDE HOOFDSTUK** beschrijft een unicenter (Universitair Ziekenhuis Gent) prospectieve cohortstudie in de adulte electieve hartchirurgie IZA omgeving. Deze tweede studie beoogde de klinische validatie van de biomerker UCHI3L1 in patiënten die electieve hartchirurgie

ondergingen. De diagnose en stagering van ANI gebeurden volgens het KDIGO systeem dat gebaseerd is op SCr en UD. De diagnose van acute tubulaire schade gebeurde volgens de UNGAL score ontwikkeld voor gebruik bij volwassen hartchirurgie patiënten. De biomerkers UCHI3L1, UNGAL en SCr werden meer frequent gemeten dan SCr in routine vroeg-postoperatieve IZA praktijk. Van de 211 gerekruteerde electieve hartchirurgie patiënten, werden 203 patiënten die zowel preoperatief als bij postoperatieve IZA opname **(t1)** geen ANI hadden, geïncludeerd in de analyse van het primaire eindpunt (i.e., ANI stadium ≥ 1 binnen de 48 u na t1), terwijl 210 patiënten die zowel preoperatief als op t1 geen ANI stadium ≥ 2 hadden, geïncludeerd werden in de analyse van het secundaire eindpunt (i.e., ANI stadium ≥ 2 binnen de 12 u na t1). Binnen de 48 u na t1 hadden 95 patiënten (46.8 %) ANI ontwikkeld, waarvan er 67 (70.5 %) stadium 1 hadden, 19 (20.0 %) stadium 2 en 9 (9.5 %) stadium 3. ANI werd hoofdzakelijk geklasseerd als ANI zonder acute tubulaire schade (84.6 %). Tijdens de eerste 4 uur postoperatief, voorspelde enkel SCr het optreden van ANI binnen de 48 u na t1, waarbij de kans dat SCr een willekeurig gekozen ziek / niet-ziek paar correct rangschikte gebaseerd op hun gemeten concentraties, hoog was. In dezelfde periode voorspelden zowel UCHI3L1 als UNGAL het optreden van ANI stadium ≥ 2 binnen de 12 u na t1. Hierbij was de kans dat hetzij UCHI3L1, hetzij UNGAL een willekeurig gekozen ziek / niet-ziek paar correct rangschikte gebaseerd op hun gemeten concentraties, echter laag. Daarenboven was deze testprestatie inferieur aan zowel die van SCr als die van de absolute verandering in SCr van de preoperatieve meting tot vroege postoperatieve metingen. Deze studie liet toe te besluiten dat in de adulte electieve hartchirurgie IZA omgeving UCHI3L1 een klinisch gevalideerde biomerker is voor de vroege diagnose of predictie van ANI stadium ≥ 2 optredend binnen de 12 u na zijn meting. De biomerker UCHI3L1 liet hierbij echter een ontoereikende klinische testprestatie zien. Dit opvallend verschil met de eerste klinische studie zou kunnen verklaard worden door de bevinding dat ANI hoofdzakelijk geklasseerd werd als ANI zonder acute tubulaire schade.

In het laatste **VIJFDE HOOFDSTUK** worden de resultaten van beide klinische studies besproken en worden belangrijke obstakels voor de bruikbaarheid van nieuwe biomerkers voor ANI diagnose alsook de vooruitzichten voor verder onderzoek toegelicht.

Curriculum Vitae

Jorien De Loor werd geboren op 25 september 1987 te Ninove. Na het beëindigen van het secundair onderwijs – richting Wetenschappen-Wiskunde – aan het Sint-Aloysiuscollege van Ninove, is zij in 2005 gestart met de studies Diergeneeskunde aan de Universiteit van Gent. Ze behaalde in 2011 het Masterdiploma van 'Veterinary Medicine in de diergeneeskunde – afstudeerrichting gezelschapsdieren' met grote onderscheiding.

In de herfst van datzelfde jaar werkte zij drie maanden als assistent bij de unit Histologie van de faculteit Diergeneeskunde, waarna ze zich in de winterperiode samen met haar promotoren engageerde voor het schrijven en indienen van een aspirantenmandaat-aanvraag bij het Fonds Wetenschappelijk Onderzoek - Vlaanderen **(FWO)** rond humane acute nierinsufficiëntie. Deze aanvraag werd in de herfst van 2012 goedgekeurd. De lente en zomer van 2012 overbrugde ze als doctoraatsbursaal aan de unit Biochemie van de faculteit Diergeneeskunde om dan aan de slag te gaan als FWO Aspirant bij diezelfde unit en de dienst Intensieve Zorg van het Universitair Ziekenhuis van Gent. Het uitgevoerde onderzoek spitste zich toe op de klinische validatie van een nieuwe kandidaat biomerker voor vroege diagnose van acute nierinsufficiëntie bij humane patiënten op Intensieve Zorg, in verderzetting van het (pre)klinisch onderzoek uitgevoerd door dr. Bert Maddens aan de unit Biochemie van de faculteit Diergeneeskunde.

Jorien De Loor is auteur van meerdere wetenschappelijke publicaties en lichtte dit onderzoek toe op verschillende nationale en internationale congressen.

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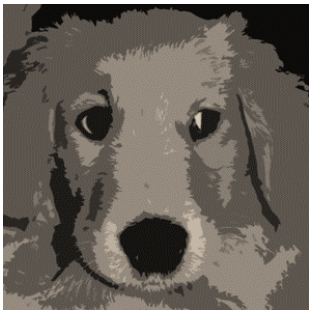

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