

# Cystatin C and neutrophil gelatinase-associated lipocalin: biomarkers for acute kidney injury after congenital heart surgery

Stefanie Seitz<sup>a,b</sup>, Manfred Rauh<sup>c</sup>, Martin Gloeckler<sup>a</sup>, Robert Cesnjevar<sup>d</sup>, Sven Dittrich<sup>a</sup>, Andreas M. E. Koch<sup>a</sup>

<sup>a</sup> Department of Pediatric Cardiology, University of Erlangen-Nuernberg, Erlangen, Germany

<sup>b</sup> Division of Pediatric Cardiology, Stollery Children's Hospital, Edmonton, Alberta, Canada

<sup>c</sup> Clinical Laboratory, University Hospital for Children and Adolescents, University of Erlangen-Nuernberg, Erlangen, Germany

<sup>d</sup> Department of Pediatric Cardiac Surgery, University of Erlangen-Nuernberg, Erlangen, Germany

## Summary

**QUESTIONS UNDER STUDY:** To evaluate the diagnostic value of serum Cystatin C and urine neutrophil gelatinase-associated lipocalin (NGAL) for the detection of acute kidney injury in patients undergoing congenital heart surgery.

**METHODS:** Serial samples of serum Cystatin C and urine NGAL were collected from 139 consecutive patients with congenital heart defects aged 3 days to 30 years after admission to the intensive care unit, 2 and 6 hours after the end of cardiopulmonary bypass. Biomarker levels were compared to perioperative data retrospectively. Acute kidney injury was defined according to the paediatric-modified RIFLE classification.

**RESULTS:** According to the paediatric-modified RIFLE criteria 53% of patients developed evidence of acute kidney injury. Serum Cystatin C concentrations were strongly correlated with severity of acute kidney injury. Optimal sensitivity of 80% and specificity of 66% for the prediction of acute kidney injury occurred at a cut-off value of 0.995 mg/l, 2 hours after the end of cardiopulmonary bypass. The 2 hour urine NGAL concentration was significantly correlated to the duration of cardiopulmonary bypass, time of aortic cross clamping, and serum lactate concentration. Moreover a significant correlation was found between urine NGAL and both length of hospital stay and mechanical ventilation.

**CONCLUSIONS:** In patients after congenital heart surgery, urine NGAL indicates the damaging force of cardiopulmonary bypass and serum Cystatin C is a valuable predictive biomarker for resulting acute kidney injury.

**Key words:** acute kidney injury; biomarker; cardiopulmonary bypass; congenital heart surgery; Cystatin C; neutrophil gelatinase-associated lipocalin

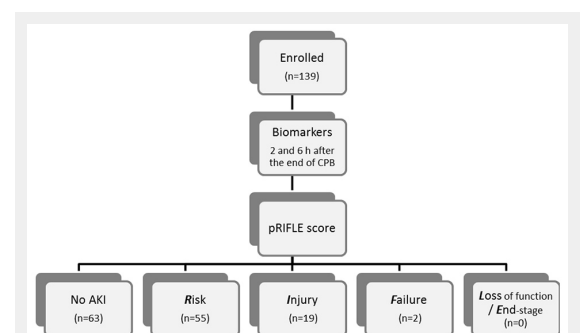
## Introduction

Acute kidney injury (AKI) is a frequent and serious complication encountered in 30–50% of adult patients after

cardiac surgery, with 1–5% needing dialysis, AKI is associated with substantial morbidity and mortality [1, 2]. Clinical investigations have identified several risk factors associated with the development of AKI after cardiopulmonary bypass (CPB). The majority related to impaired renal perfusion or decreased renal reserve [3].

In current clinical practice AKI is still diagnosed by measuring serum creatinine although serum creatinine is an inadequate marker due to its late rise in AKI pathophysiology [2]. It is a functional marker of glomerular filtration rate rather than injury, only rising once substantial renal injury has already occurred e.g. in a steady state of chronic renal failure and is therefore an unreliable indicator during acute changes in kidney function. Since creatinine formation is proportional to muscle mass and age, serum creatinine may remain within normal range in patients who are elderly, malnourished or chronically ill [4].

Back in 1985 Cystatin C was already investigated as an additional marker for glomerular filtration rate [5]. Cystatin C is a 13 kD protein produced by a housekeeping type gene by all nucleated cells. Cystatin C is freely filtered in the glomerulus and is completely taken up by the proximal tubule [5]. The independence from height, gender, age, and muscle mass is advantageous. Children and the elderly benefit in particular [6]. Numerous studies have already eval-



**Figure 1**  
Flow diagram of the study.

uated the use of serum Cystatin C as an endogenous marker of kidney function in populations at risk to develop AKI, showing, that serum Cystatin C has a superior performance in the diagnostic accuracy of serum creatinine levels in the early detection of impaired kidney function [7, 8].

More recently, several novel biomarkers discovered from animal models, have been studied in humans, indicating renal structural damage at an earlier stage to provide potentially effective therapies at a point when damage is still reversible [9–11]. Translational research has identified neutrophil gelatinase-associated lipocalin (NGAL) as one promising early biomarker for diagnosis and prediction of acute renal injury [12, 13]. The first clinical studies in children revealed increased NGAL levels in the urine and plasma by enzyme-linked immunosorbent assay two hours after cardiopulmonary bypass in subjects who subsequently developed AKI [14–17].

As we have recently demonstrated, patients with normal baseline serum creatinine levels displayed a significant correlation between plasma NGAL measured subsequently after cardiopulmonary bypass and increase in serum creatinine, supporting the role of NGAL as an early biomarker of acute renal injury [18]. However, this study revealed also the limitations of a single plasma NGAL determination in predicting AKI development [18].

Therefore, we initiated this prospective single centre study of children undergoing cardiac surgery to compare the diagnostic validity of postoperative urinary NGAL and serum Cystatin C as early biomarkers and predictors of acute renal failure.

## Patients and methods

### Study design

All patients undergoing CPB for surgical correction or palliation of congenital heart disease from December 2010 until November 2011 were prospectively enrolled. We obtained written informed consent from the legal guardian of every patient before enrolment. The study was approved by the local Ethics Committee and complied with the declaration of Helsinki. Exclusion criteria included preexisting

renal insufficiency, and patients with history of potential nephrotoxin use during the preoperative days. Within the context of the routine blood collection, we obtained blood samples within 30 minutes after admission to the Intensive Care Unit (i.e. two to three hours after the end of CPB), and another 4 hours later from an arterial line. Additionally, we obtained spot urine samples 2 and 6 hours after CPB.

Biomarker levels were compared to perioperative data retrospectively.

### Patients

A total of 139 patients (76 males and 63 females) were enrolled. Age at surgery was 1 day to 44.3 years (mean age 4.8 years, median age 0.9 years, interquartile range [ICR] 0.3–7.7 years). The overall patients comprised of 24 newborns, 49 infants, 46 children, and 20 adolescent or adult patients. For the underlying congenital heart defects see table 1.

### Serum Cystatin C

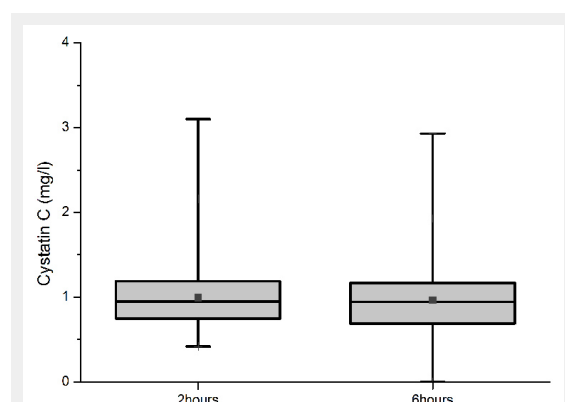
Blood samples were centrifuged and the plasma aliquoted for storage at  $-80^{\circ}\text{C}$ . Cystatin C was measured using an immunonephelometric method (BN ProSpec-II, Siemens Healthcare Diagnostics) [19].

### Urine NGAL

Urine samples were centrifuged and the supernatants stored in tubes at  $-80^{\circ}\text{C}$ . NGAL concentrations were measured by a two-step sandwich assay using chemiluminescent microparticle immunoassay technology (ARCHITECT analyser, Abbott Diagnostics) [17]. High affinity antibodies were generated toward distinct epitopes on NGAL and assay standards were prepared using human recombinant NGAL. Urine NGAL concentrations were measured using the standardised manufacturer's protocol in the range of 0–1,000 ng/ml. Serial dilution was performed where measured values exceeded this range.

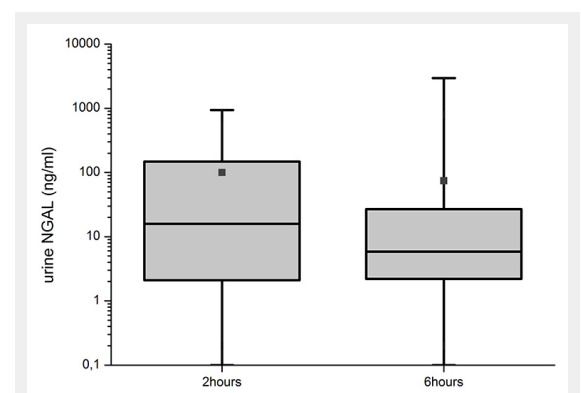
### Additional laboratory data

Serum creatinine was measured routinely at the time of preoperative evaluation and shortly after surgery (at the same



**Figure 2**

Box and whisker plot of serum Cystatin C concentrations 2 hours and 6 hours after the end of cardiopulmonary bypass. Boxes: 25<sup>th</sup> percentile (bottom line), median (middle line), and 75<sup>th</sup> percentile (top line); bars: range; squares: means.



**Figure 3**

Box and whisker plot of urine neutrophil gelatinase-associated lipocalin (NGAL) values in all patients 2 hours and 6 hours after the end of cardiopulmonary bypass. Boxes: 25<sup>th</sup> percentile (bottom line), median (middle line), and 75<sup>th</sup> percentile (top line); bars: range; squares: means. Note the logarithmic scale.

time as the first postoperative Cystatin C measurements). Table 2 gives the normal values of serum creatinine in our laboratory. Estimated creatinine clearance (eCrCl) was calculated using the Schwartz formula:  $eCrCl \text{ (ml/min/1.73 m}^2\text{)} = \text{height (cm)} \times k/SCr$  (with  $k = 0.45$  in the first year of life,  $k = 0.55$  in infants and children,  $k = 0.7$  in male patients  $>12$  years).

#### Reference standard and clinical variables

The primary outcome variable was the development of AKI according to the RIFLE criteria. RIFLE is an acronym for **R**isk for renal injury, **I**njury to the kidney, **F**ailure of kidney function, **L**oss of kidney function, and **E**nd-stage renal disease. All patients were classified using the paediatric modified criteria of RIFLE [20]. This scheme stages renal function from risk through to end-stage renal failure based on serum creatinine/glomerular filtration rate or urine output. In practice, a decrease of estimated creatinine clearance of at least 25%, 50% or 75% leads to pRIFLE scores of R, I or F, respectively. Persistent renal failure for more than four weeks or three months indicates classification L or E, respectively [20]. The patients were classified according to the pRIFLE score once a day during the stay in the intensive care unit.

All laboratory measures were interpreted blind to the pRIFLE score.

Other variables included: age, gender, cardiopulmonary bypass time, aortic cross clamping time, length of mechanical ventilation and length of stay in the intensive care unit.

#### Statistics

The associations of urine NGAL and serum Cystatin C to continuous variables were assessed by univariate regression analysis. The association of urine NGAL and Cystatin C to the pRIFLE scores was assessed by Spearman's correlation coefficient. Statistical differences between the two sexes, and between pRIFLE grades were evaluated using the rank score test on unpaired data (Wilcoxon rank-sum test). P-values were two-tailed, with a value  $<0.05$  considered to be significant and  $<0.01$  considered to be highly significant.

Receiver operating characteristic (ROC) curves were computed to assess whether Cystatin C and urine NGAL could predict the risk of developing different pRIFLE scores. The sensitivity and specificity for the biomarkers at varying cut-off levels were calculated by calculating the area under the ROC curve. The best cut-off level was defined as the value that is closest to the point on the ROC curve where sensitivity and specificity approach unity.

All data was analysed with SPSS 18.0 for Windows.

#### Results

Figure 1 summarises the design of the study. Perioperative data of the 139 patients included in the study are given in table 3.

#### Kidney function

According to the pRIFLE criteria 63/139 (45%) patients developed no evidence of acute kidney injury. However, 55/139 (40%) patients and 19/139 (14%) patients reached a pRIFLE max of R and I, respectively. A total of 2 patients

**Table 1:** Cardiac diagnosis of the patient group.

Heart defect	n
Functional univentricular heart of all stages	19
Ventricular septal defect	18
Atrial septal defect	17
Transposition of the great arteries	12
Pulmonary atresia with VSD	11
Tetralogy of Fallot	11
Aortic stenosis (subaortic, valvular, supravalvular)	11
Atrioventricular septal defect	9
Coarctation/Interruption of the aortic arch	9
Double outlet right ventricle and (complex forms)	4
Truncus arteriosus communis	4
Pulmonary artery stenosis	3
Vascular ring	3
Mitral valve insufficiency	3
Total anomalous pulmonary venous drainage	2
Pulmonary atresia with intact ventricle septum	2
Ebstein anomaly	1

**Table 2:** Normal values of serum creatinine.

Age	Upper limit of the normal range (mg/dl)
<60 days	0.88
60–360 days	0.39
1–2 years	0.35
3–4 years	0.42
5–6 years	0.47
7–8 years	0.53
9–10 years	0.64
11–12 years	0.68
13–14 years	0.77
>15 years (female)	0.95
>15 years (male)	1.17

(1.4%) were in the “Failure“ category and underwent dialysis. No patient reached a pRIFLE max of L or E.

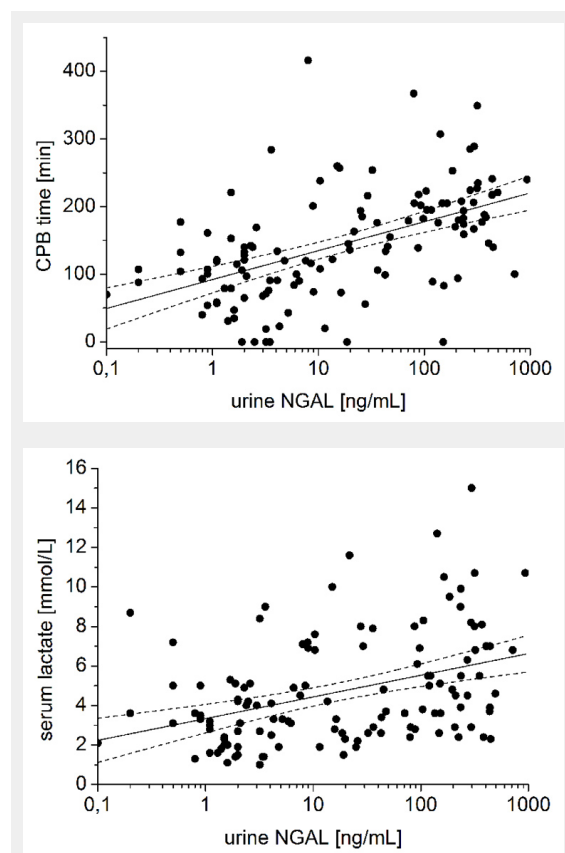
All patients had normal serum creatinine levels according to sex and age before surgery between 0.16 and 0.89 mg/dl (mean 0.38 mg/dl, median 0.33 mg/dl, interquartile range [IQR] 0.26–0.46 mg/dl).

Serum creatinine increased in all patients within 72 hours to peak levels between 0.22 and 2.01 mg/dl (mean 0.58, median 0.49, IQR 0.37–0.73 mg/dl), the serum creatinine increase from baseline to peak value was between 0 and 1.12 mg/dl (mean 0.20, median 0.13, IQR 0.06–0.22 mg/dl). 22/139 patients (15%) had an increase of >50%.

The preoperative estimated creatinine clearance was between 19 and 202 ml/min/1.73 m<sup>2</sup> (mean 114, median 116, IQR 84–141 ml/min/1.73 m<sup>2</sup>). The postoperative creatinine clearance was between 13 and 153 ml/min/1.73 m<sup>2</sup> (mean 80, median 78, IQR 48–110 ml/min/1.73 m<sup>2</sup>). The estimated creatinine clearance decreased from 114 ± 44 to 80 ± 36 ml/min/1.73 m<sup>2</sup> within the first 18 hours after surgery.

### Serum Cystatin C

Serum Cystatin C concentration in the first (fig. 2) and second sample was between 0.42 and 3.25 mg/l (mean 0.99, median 0.95, IQR 0.75–1.19 mg/l; n = 139) and between 0.42 and 3.2 mg/l (mean 0.96, median 0.94, IQR 0.69–1.17 mg/l; n = 126), respectively.



**Figure 4**

Correlations between urine NGAL 2 hours after the end of cardiopulmonary bypass (CPB) and (a) duration of CPB ( $P < 0.01$ ), and (b) serum lactate concentration ( $P < 0.01$ ). Solid and dotted lines represent the regression line and the 95% confidence interval, respectively.

Serum Cystatin C levels began to increase 2 hours after the end of CPB and became only marginally higher after 6 hours after the end of CPB. Serum concentrations 2 hours and 6 hours after the end of CPB showed highly significant correlation with peak postoperative serum creatinine, absolute and relative increase in serum creatinine, estimated creatinine clearance postoperatively and the pRIFLE score ( $P < 0.01$ ). In addition, serum Cystatin C concentrations 2 hours and 6 hours after the end of CPB strongly correlated with length of hospital stay and mechanical ventilation ( $P < 0.01$ ).

To determine the ability of serum Cystatin C to predict AKI the ROC curves were generated for each time-point after CPB (2 and 6 hours). AUC were 0.741 (95% CI: 0.606 to 0.875) for the 2-hour Cystatin C ( $P = 0.003$ ) and 0.707 (95% CI: 0.543 to 0.871) for the 6-hour Cystatin C ( $P = 0.009$ ). Further analysing the ROC curves demonstrate the superiority of 2 hour serum Cystatin C for the detection of AKI with an optimal sensitivity and specificity of using a cut-off value of 0.995 mg/l (fig. 5, table 4).

### Urine NGAL

Urine NGAL concentration in the first (fig. 3a) and second sample (fig. 1b) was between 0 and 940 ng/ml (mean 100, median 16, IQR 2–149 ng/ml; n = 125), and between 0 and 795 ng/ml (mean 74, median 6, IQR 2–127 ng/ml; n = 114), respectively. There were no differences in urine NGAL levels between females and males, and there were no correlations between urine NGAL and age, body weight, or body height.

However, highly significant correlations between the first urine NGAL sample and the duration of cardiopulmonary bypass ( $P < 0.01$ ; fig. 4a), the time of aortic cross clamping ( $P < 0.01$ ), serum lactate concentration ( $P < 0.01$ ; fig. 4b) and estimated creatinine clearance postoperatively ( $P < 0.01$ ) were displayed. Furthermore, the first and second urine sample were both correlated to length of hospital stay ( $P < 0.05$ ) and time of mechanical ventilation ( $P < 0.05$ ).

No correlations were demonstrated between urine NGAL and peak postoperative serum creatinine, absolute and relative increase in serum creatinine, and the pRIFLE score.

### Discussion

The aetiology of AKI after CPB is multifactorial and incompletely understood. Possible aetiologies include ischaemia-reperfusion injury, oxidative stress, and the systemic inflammatory response. All these factors may contribute to endothelial dysfunction, resulting in capillary leak and vasomotor instability [14].

Serum creatinine is a well established parameter for kidney function despite its generally recognised limitations as an ideal marker for GFR especially in children and elderly. Recently, experimental research has proposed several novel promising renal biomarkers indicating renal tubular injury [9, 10, 21, 22]. However, none of these markers has yet been successfully implemented in routine clinical practice to replace serum creatinine.

Cystatin C has already been recognised to be a superior marker to serum creatinine in the assessment of impaired kidney function in numerous studies. These findings re-

vealed that a rise in Cystatin C precedes the increase in serum creatinine in a select population at high risk for development of AKI [6–8, 23]. Cystatin C is mainly a marker for GFR, serum concentration might only rise after the rate begins to fall. Particularly in children, the independence from: height, gender, age and skeletal muscles, is advantageous [6].

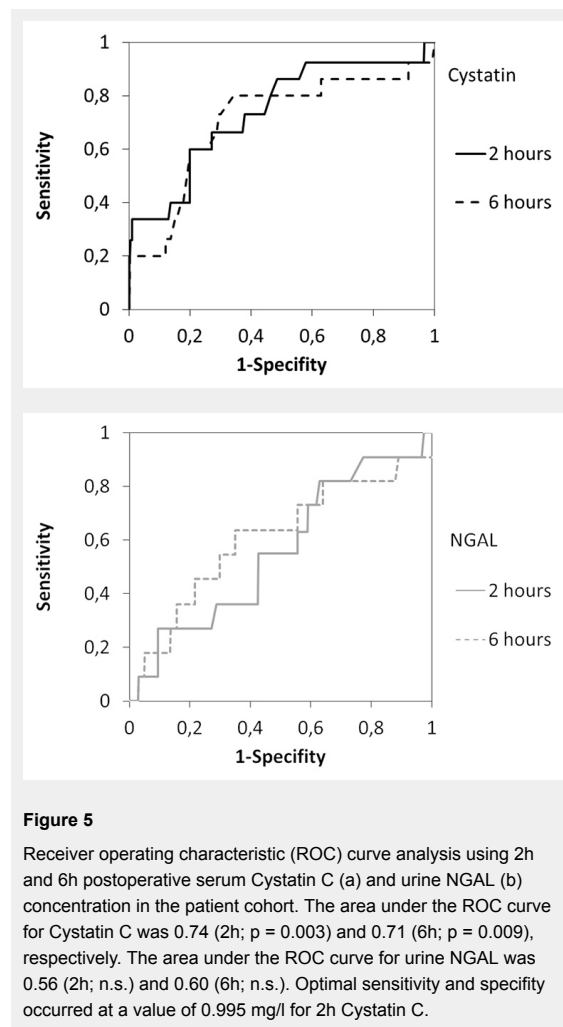
Many studies have focused on the diagnostic predictive value of serum Cystatin C levels for AKI. These studies demonstrated the superiority of serum Cystatin C com-

pared to serum creatinine as a useful biomarker for the early prediction AKI. These findings were confirmed by a systemic review and meta-analysis of diagnostic test studies on the predictive value of Cystatin C in populations at risk for AKI [24].

Most of these studies on Cystatin C were obtained on adult patients [7, 23–25], only two studies investigated paediatric patients after cardiac surgery [19, 26]. The first clinical study in children indicated a significant increase of serum Cystatin C after CPB. Maximal sensitivity and specificity for prediction of AKI were described 12 hours after CPB. The 12 hours Cystatin C also correlated with severity and duration of AKI as well as length of hospital stay [19]. The second multicentre study of 288 children undergoing cardiac surgery evaluated the predictive value of Cystatin C compared to serum creatinine [26]. This study revealed that Cystatin C levels obtained within the first 6 hours of surgery strongly predicts stage 1 and 2 AKI development in the subsequent days as well as longer duration of mechanical ventilation and longer hospital stay [26].

In our single centre study of children undergoing cardiac surgery, the results are consistent with the findings of these two former paediatric studies [19, 26]. We also found that serum Cystatin C performs well as a marker to detect early AKI following paediatric CPB. However, at first glance our results seem to be contradictory to the findings of the Cincinnati study group regarding the optimal time of Cystatin C measurement. According to their data the Cincinnati study group suggest the 12 hour value of Cystatin C as the optimal threshold and a cut-off value of 1.16 mg/l [19]. In contrast, our data revealed an optimal time point for the prediction of AKI at a Cystatin C concentration of 0.995 mg/l, already 2 hours after the end of CPB with adequate certainty. The 2 hour Cystatin C also strongly correlated with severity and duration of AKI as well as length of hospital stay, and mechanical ventilation. Looking in detail at the study design of the two reports, the results are less conflicting. The Cincinnati study group collected their serum samples 2, 12, and 24 hours after the initiation of CPB, whereas we timed the sample collection after the termination of CPB.

Moreover, only a few studies on NGAL measurements in children after CPB have been performed so far, most of



**Table 3:** Baseline data of the patients (n = 139; males/females = 1.2).

	Median [IQR]
Age, years	0.9 [0.3–7.7]
Height, cm	72 [57–118]
Weight, kg	8 [5–22]
Cardiopulmonary bypass, min	138 [87–195]
Aortic cross clamping, min	59 [24–110]
Serum lactate, mmol/L	3.6 [2.6–6.3]
Mechanical ventilation, hours	28 [11–153]
ICU, days	4.5 [2.3–10.0]

**Table 4:** Sensitivity, specificity and accuracy for the patient cohort at the optimal serum Cystatin C and urine NGAL concentration.

	Cutt-off-value	Sensitivity	1-Specificity	Accuracy
Cystatin C, 2 hour level	0.995 mg/l	0.800	0.342	0.65
Cystatin C, 6 hour level	0.945 mg/l	0.800	0.459	0.58
Urine NGAL, 2 hour level	27.6 ng/ml	0.545	0.426	0.55
Urine NGAL, 6 hour level	10.4 ng/ml	0.636	0.351	0.64

them were attained by the Cincinnati study group. The results of these studies have provided evidence, that plasma NGAL and urine NGAL concentrations rise substantially in children after CPB and furthermore proved to be significant predictive biomarkers for AKI and its clinical outcome [14–16]. Recently, we evaluated the diagnostic value of plasma NGAL in routine clinical practice for the detection of AKI [18]. Our results identified significant correlations between plasma NGAL and an increase in postoperative serum creatinine; however, a marked overlap between the patient groups and only a marginal predictive value depicted the limitations of a single NGAL determination in the effective determination of AKI [18].

In our present study we focused solely on the measurement of urine NGAL. Correspondingly, we found significant correlations between the increase of urine NGAL two hours after CPB and the duration of CPB, the time of aortic cross clamping, serum lactate concentration and estimated creatinine clearance postoperatively. Our findings are consistent with previous clinical studies on NGAL measurements in children after CPB where significant correlations have been found between acute renal ischemia-reperfusion injury and both serum and urine NGAL [14–16, 18]. However, while former studies have detected predictive values, the regression coefficient of the positive correlation in our patients was small, and there was a spread of individual values. Therefore, it was not possible to estimate conclusively the subsequent increase in serum creatinine from one single postoperative urine NGAL level. Correspondingly, we were not able to generate a reasonable optimal cut-off value for urine NGAL. Moreover, no significant correlation was detected between urine NGAL and the severity of the pRIFLE score. These findings albeit less distinctive than our previous data imply that neither urine nor plasma NGAL were accurate predictive biomarkers for AKI. Nevertheless, the significant correlations in our study emphasise that NGAL is indeed an effective early marker of acute ischaemic renal injury in accordance with studies on NGAL in early post-ischaemic mouse kidneys in which an up regulation of NGAL messenger RNA in the thick ascending limb of Henle's loop and the collecting ducts resulted in the synthesis of NGAL protein levels in the distal nephron and the secretion in the urine have been demonstrated [12, 27]. Furthermore, a marked amelioration of ischaemic acute renal injury by intravenous administration of recombinant NGAL has been discovered [28].

In summary, our findings indicate, that urine NGAL is a valuable immediate biomarker for acute renal injury in paediatric patients undergoing cardiac surgery, preceding the increase in serum creatinine by 1–3 days. The determination of serum Cystatin C provides an efficient diagnostic device to detect the development and characterise the severity of an early deterioration of kidney function initiated by the ischaemic injury. The diagnosis of AKI can be assumed at a Cystatin C concentration of 0.995 mg/l, 2 hours after the end of CPB. In routine clinical practice both biomarkers each have a limited predictive value due to their spread distribution, but Cystatin C determination seems to have a major impact for the individual patient by predicting AKI. Additional measurement of urine NGAL, however, helps to assess kidney injury.

**Funding / potential competing interests:** Reagents for measuring urine NGAL were provided free of cost by Abbott Diagnostics.

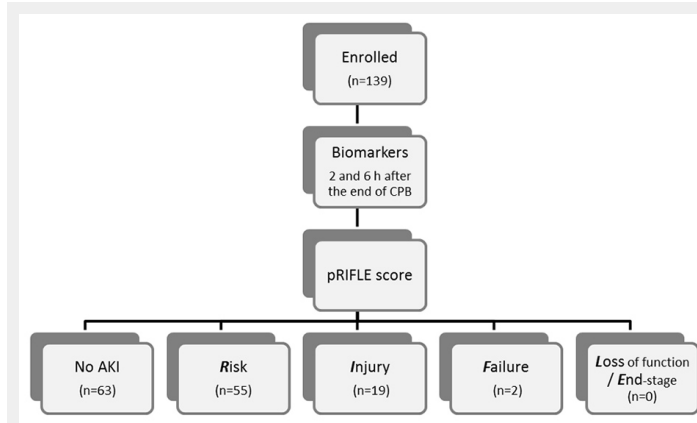
**Correspondence:** Professor Andreas Koch, MD, Department of Pediatric Cardiology, University Hospital for Children and Adolescents, Loschgestr. 15, DE-91054 Erlangen, Germany, [andreas.koch\[at\]juk-erlangen.de](mailto:andreas.koch[at]juk-erlangen.de)

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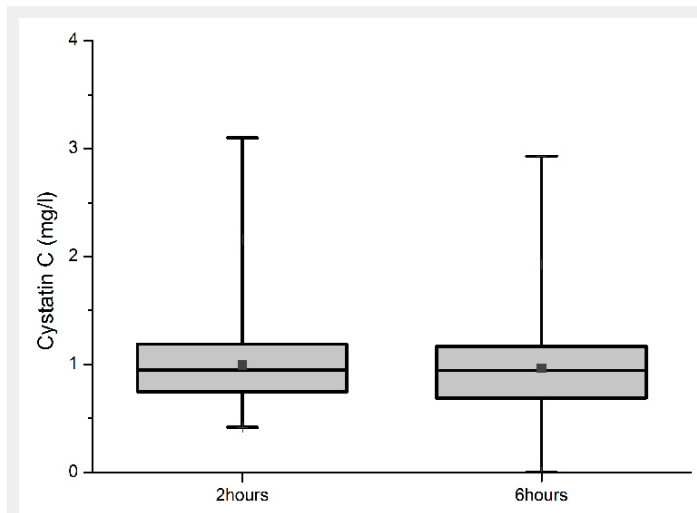
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Figures (large format)

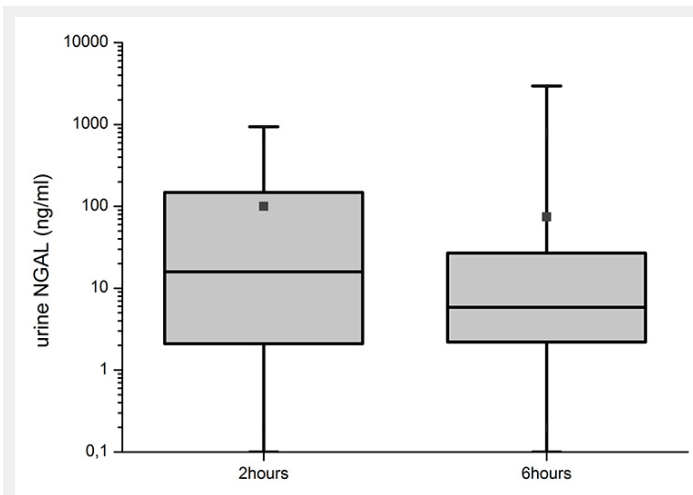


**Figure 1**  
Flow diagram of the study.



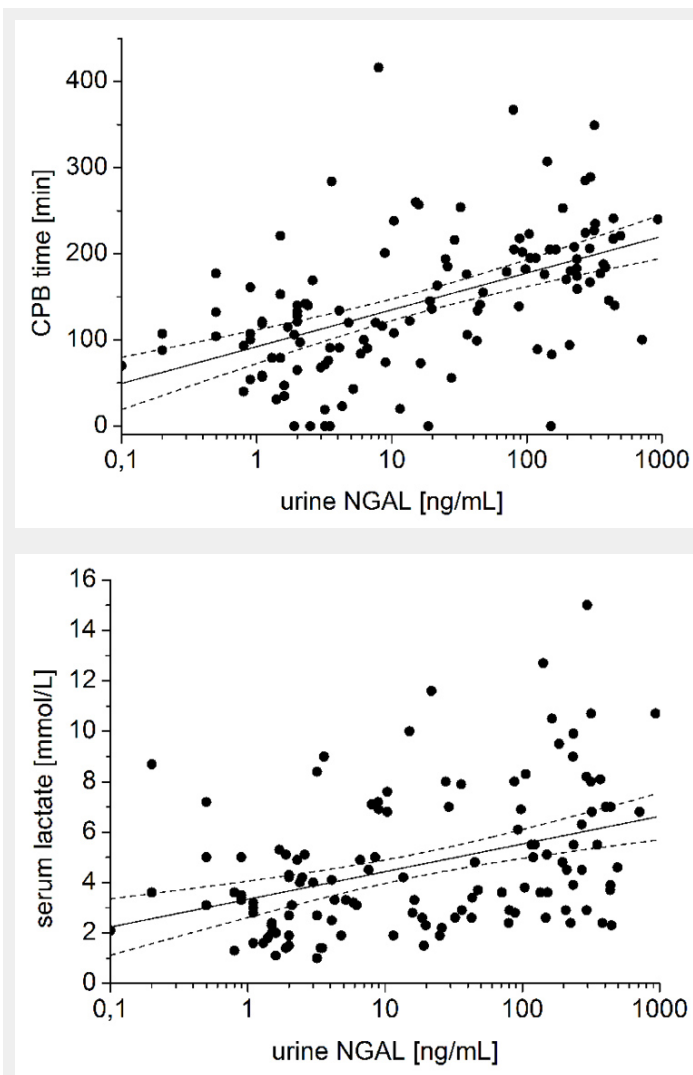
**Figure 2**  
Box and whisker plot of serum Cystatin C concentrations 2 hours and 6 hours after the end of cardiopulmonary bypass. Boxes: 25<sup>th</sup> percentile (bottom line), median (middle line), and 75<sup>th</sup> percentile (top line); bars: range; squares: means.





**Figure 3**

Box and whisker plot of urine neutrophil gelatinase-associated lipocalin (NGAL) values in all patients 2 hours and 6 hours after the end of cardiopulmonary bypass. Boxes: 25<sup>th</sup> percentile (bottom line), median (middle line), and 75<sup>th</sup> percentile (top line); bars: range; squares: means. Note the logarithmic scale.



**Figure 4**

Correlations between urine NGAL 2 hours after the end of cardiopulmonary bypass (CPB) and (a) duration of CPB ( $P < 0.01$ ), and (b) serum lactate concentration ( $P < 0.01$ ). Solid and dotted lines represent the regression line and the 95% confidence interval, respectively.

