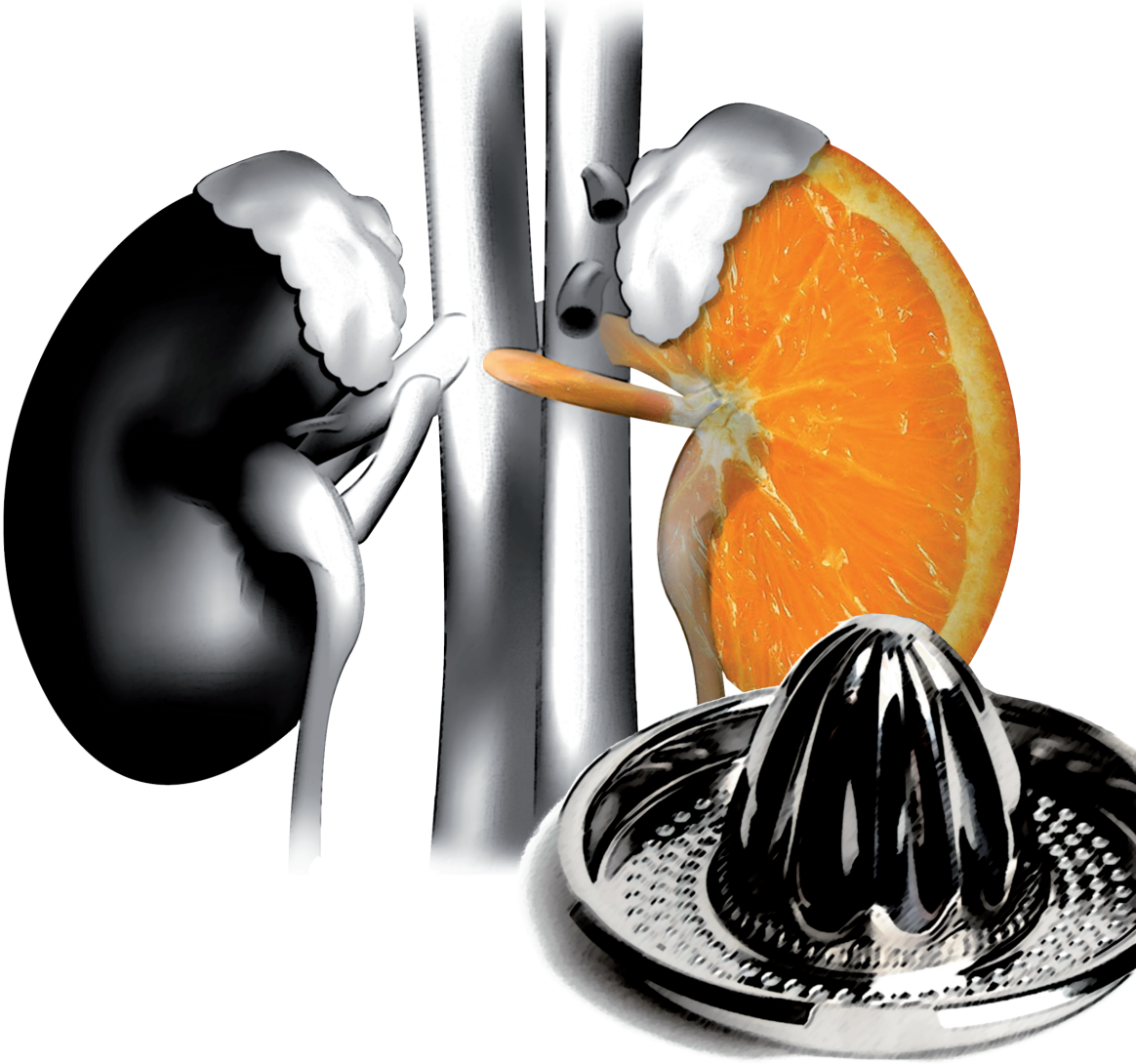

Do not squeeze the kidneys!

Acute Kidney Injury in Critically Ill Children

Alexandra J.M. Zwiers

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Acute Kidney Injury in Critically Ill Children

Do not squeeze the kidneys!

Acute nierschade in kritisch zieke kinderen

Wring de nieren niet uit!

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.

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Dr. S.N. de Wildt

Het mooiste dat het leven biedt
Zijn af en toe de dromen,

Dat alles nog beginnen moet
En 't beste nog moet komen.

Toon Hermans

Voor oma Mia

CONTENTS

PART I

INTRODUCTION

1 General introduction

Adapted from:
Biomarkers and clinical tools in critically ill children: are we heading toward tailored drug therapy?

Erik A.B. Buijs, Alexandra J.M. Zwiers, Erwin Ista, Dick Tibboel, Saskia N. de Wildt

Biomarkers in Medicine 2012, Jun;6(3):239-57

ACUTE KIDNEY INJURY

Acute kidney injury (AKI) (previously called acute renal failure) is characterized by the abrupt inability of the kidneys to adequately excrete waste products and regulate fluid and electrolyte homeostasis appropriately. This results in an at least partially reversible increase in the blood concentration of creatinine and nitrogenous waste products (1). Renally eliminated medication will accumulate, and nephrotoxic drugs may provide a "second hit" to the already injured kidneys. Furthermore, fluid management and nutrition will be hampered by oliguria. AKI is typically classified as pre-renal, intrinsically renal, or post-renal (2).

Pre-renal AKI may occur as a consequence of a reduced renal blood flow due to several conditions leading to intravascular volume depletion and/or compromised cardiac output (3). Since the kidneys are intrinsically normal, pre-renal injury is considered reversible once the hemodynamic conditions have been restored to normal. When pre-renal injury persists for a longer period of time, hypoperfusion of the kidneys will lead to hypoxic or ischemic acute tubular necrosis (ATN), a form of intrinsic AKI. During the evolution of pre-renal to intrinsically renal injury several compensatory pathways in the kidney are activated to maintain renal perfusion (4). These compensatory pathways include intra-renal generation of vasodilatory prostaglandins and angiotensin II, which increases efferent arteriolar resistance, thereby increasing intraglomerular pressure to maintain or even raise the glomerular filtration rate. There are, however, certain clinical circumstances (e.g., administration of cyclo-oxygenase inhibitors, angiotensin converting enzyme inhibitors or nephrotoxic drugs) that may inhibit or interfere with one of the compensatory mechanisms and thus precipitate AKI (1, 4).

In intrinsic AKI, the pathology lies within the kidney itself. In the critically ill patient it is often caused by ATN or interstitial nephritis, elicited by a wide range of drugs or infectious agents. Other causes are glomerulonephritis and hemolytic uremic syndrome. Post-renal AKI is caused by an obstruction of the urinary outflow tract such as intraluminal obstruction due to urethral valves or coagulated blood, functional obstruction due to a neurogenic bladder or extraluminal obstruction which may result from malignant conditions (2). This form of AKI can be restored by removal of the actual obstruction or by insertion of a urinary deviation proximal to the obstruction. Altogether, AKI occurs with variable severity and in many clinical scenarios.

Clinical consequences

AKI requires adjustment of treatment in the short-term; for example, adjustment of dosages of renally eliminated drugs, avoidance of nephrotoxic drugs, and correction of possible electrolyte and acid-base imbalances. In case of oliguric or anuric AKI, physicians shift focus to maintaining a neutral fluid balance, especially when hypertension

develops due to intravascular volume expansion or activation of the renin-angiotensin system.

Maintaining neutral fluid balance remains a significant challenge, however, because in critically ill patients with or at risk for AKI fluid replacement and fluid overload must be delicately balanced. In this light, Goldstein proposed a strategy of fluid management based on a conceptual model in which three phases are distinguished according to the clinical status: [1] fluid resuscitation/repletion, [2] fluid balance maintenance, and [3] fluid removal/recovery (5). In this strategy, volume and timing of each phase are deemed critical (5). Still, despite optimal fluid care renal replacement therapy (RRT) may be needed to correct fluid, electrolyte, and acid-base balances, and to enable adequate feeding until renal function improves.

Outcomes

The impact of AKI on clinical outcomes has been underappreciated for a long time. It has traditionally been considered a surrogate marker for severity of illness, and patient mortality was considered a consequence of the underlying disease (6). However, there is increasing evidence that AKI itself is associated with poor outcomes including longer length of pediatric ICU stay, prolonged duration of mechanical ventilation, and pediatric ICU mortality (7, 8). These associations further emphasize the importance of timely detection and management of AKI.

Apart from the short-term consequences, severe AKI can result in a substantial permanent loss of functioning nephrons, which may lead to hyperfiltration in the remaining nephrons in the long-term (9-11). This compensatory response will initially maintain glomerular filtration rate (GFR) but may progress to interstitial fibrosis and tubular atrophy. These changes may eventually lead to chronic kidney disease (CKD), a condition characterized by a progressive decline in GFR over time, proteinuria, and/or systemic hypertension (12, 13). Early detection of CKD is especially important in children since, despite initial recovery of GFR, renal injury may only become evident during growth when the number of intact nephrons relative to body mass decreases. Also, timely therapeutic interventions can slow the progression of CKD while medication should be dosed adjusted to actual clearance (14, 15).

The few follow-up studies in survivors of pediatric AKI report prevalences of CKD from 6% to 59% (16-21). The wide-spread incidences in these studies may be partly explained by the large heterogeneity of AKI and CKD definitions, the inclusion of patients with AKI of varying causes including pre-existing renal disease, and small sample sizes. In 2005 Hui-Stickle and colleagues were one of the first to report that 34% of 176 pediatric AKI survivors had a reduced kidney function, some were even dialysis dependent, upon discharge from a tertiary center (22). Askenazi and colleagues followed this specific cohort for 3 to 5 years and found that no more than 57% had survived, and that most deaths

had occurred within 2 years of the AKI episode. Moreover, 17 of 29 patients (59%) studied during a follow-up clinic visit demonstrated evidence of CKD, manifesting as hyperfiltration, reduced kidney function, hypertension, or microalbuminuria (16). Given that the definition of CKD used by Askenazi and colleagues included hyperfiltration, this may in part explain the high incidence (16). The opposite holds true for the prospective study of Mammen and colleagues. Using a more strict CKD definition (albuminuria and/or eGFR <60 mL/min per 1.73m^2) they reported a considerably lower incidence of 10% among 126 patients surviving AKI in the pediatric ICU at a median follow-up of approximately one year (18). However, this relatively short follow-up period potentially underestimates the true incidence burden of CKD (23, 24). Nonetheless, all studies clearly indicate that survivors of AKI during childhood are at risk of residual renal injury.

Definitions and diagnostic methods of AKI

Reported incidences of AKI in critically ill children admitted to an ICU vary from 4.5% to 82.0% (7, 8, 25-28). This wide range is partly explained by the lack of a consensus definition of AKI. In fact, over the past years, more than 35 different definitions have been used to define AKI (29). In 2004 the Acute Dialysis Quality Initiative (ADQI) group for the study of AKI, composed of nephrologists and intensivists, proposed an empiric working AKI definition, the RIFLE (acronym for Risk, Injury, Failure, Loss of kidney function, and End-stage kidney disease) classification (30). The RIFLE categories are based on the relative change in serum creatinine (SCr) level combined with urinary output (UO) and three levels of AKI severity (Risk, Injury, and Failure) and two outcome classes (Loss of kidney function and End stage kidney disease) are distinguished (Table 1). In 2007, experts in the Acute Kidney Injury Network (AKIN) proposed a modified version of the RIFLE classification (31). This version used the terms mild (stage 1), moderate (stage 2) and severe AKI (stage 3) analogous to the RIFLE categories Risk, Injury and Failure. The major differences from the RIFLE classification include a smaller change in SCr (exceeding ≥0.3mg/dL or ≥26.2 μmol/L) within 48 hours as the AKI threshold versus SCr 1.5x baseline, and the use of either SCr or UO. In both classifications patients receiving RRT were classified as AKIN stage 3 or Failure. For children, Akcan-Arikan and colleagues proposed an adapted RIFLE classification (26). The main difference is a lower cut-off SCr to achieve the F category to reflect that, because children have a lower baseline SCr, a SCr of 4.0 mg/dl is not needed to cause severe dysfunction (32). Subsequent studies in hospitalized adults and children showed that the (p)RIFLE- and AKIN classification allowed for the detection of AKI across different clinical settings, helps in generalization of data generated from single center studies, classified AKI severity, and was an independent risk factor for morbidity and mortality (7, 27, 28, 33-37).

Still, one must realize that all classification systems rely on conservative measures of renal function or GFR: changes in SCr and UO. In general, the ideal marker for mea-

Table 1. Description of RIFLE, AKIN, and pRIFLE definition criteria for AKI
AKI, acute kidney injury; AKIN, Acute Kidney Injury Network; eGFR, estimated glomerular filtration rate; RIFLE, Risk–Injury–Failure–Loss–End stage renal disease; RRT, renal replacement therapy; SCr, serum creatinine; UO, urinary output.

Definition System						
RIFLE (30)	**Class R (Risk)**		**Class I (Injury)**		**Class F (Failure)**	
	SCr increase ≥1.5 fold *or* eGFR decrease >25% compared to baseline	UO < 0.5 ml/kg/hr x 6h	SCr increase ≥2-fold or eGFR decrease >50% compared to baseline	UO < 0.5 ml/kg/hr x 12h	SCr increase ≥3-fold or eGFR decrease >75% compared to baseline *or* SCr ≥4 mg/dL combined with increase of ≥0.5 mg/dL	UO < 0.3 ml/kg/hr x 24h *or* anuria x 12h
AKIN (31)	**Stage I**		**Stage II**		**Stage III**	
48-hour moving window	SCr increase ≥1.5-fold *or* ≥0.3 mg/dL	UO < 0.5 ml/kg/hr x 6h	SCr increase ≥2-fold	UO < 0.5 ml/kg/hr x 12h	SCr increase ≥3-fold compared to baseline, *or* SCr ≥4 mg/dL combined with an increase of ≥0.5 mg/dL, or RRT	UO < 0.3 ml/kg/hr x 24h *or* anuria x 12h
pRIFLE (26)	**Class R (Risk)**		**Class I (Injury)**		**Class F (Failure)**	
	eGFR decrease > 25%	UO < 0.5 ml/kg/h x 8h	eGFR decrease >50%	UO < 0.5 ml/kg/h x 16h	eGFR decrease > 75% *or* eGFR <35 ml/min/1.73m^2	UO < 0.3 ml/kg/h x 24h *or* anuria x 12h

suring GFR should be physiologically inert, freely filtered in the glomerulus, neither secreted nor reabsorbed by the renal tubule, and not synthesized or metabolized by the kidney. Inulin, an exogenous polysaccharide, is one of these substances, and has all these properties. Consequently, blood plasma clearance of inulin is considered the traditional gold standard for measuring GFR, both in adults and in children (11). During this method inulin is intravenously administered, either as a continuous infusion or by a single bolus injection, after which blood samples are collected at specific time-intervals (38, 39). The GFR is estimated from the inulin values at steady state with the continuous infusion method or from the concentration-time curve after the single bolus injection. Unfortunately, the inulin clearance methods have several disadvantages: it is time-consuming for both patients and clinicians, and intravenous access as well as repeated blood sampling is required which may be particularly cumbersome in pediatric practice (38). An alternative method is the use of radionuclides including 125I-iothalamate, 99mTc-diethylenethiaminepenta-acetic acid and 51Cr-ethylenediaminetetra-acetic acid (11). The use of these isotopic markers is limited, however, due to the high costs, the radioactive exposure as well as requirements for appropriate handling and disposal of radioactive materials including the patient's urine specimens (40). So far, in daily clinical practice SCr still forms the cornerstone in the estimation of GFR, for which many algorithms have been developed (41-45).

Creatinine has a molecular weight of 113 Da and is a compound derived from the metabolism of creatine phosphate in muscles and dietary meat intake. It is released into the plasma at a relatively constant rate, not bound to plasma protein, and freely filtered by the glomerulus. Creatinine is not reabsorbed in the tubules or metabolized by the kidney (46). Though it seems an ideal marker of GFR, the use of SCr has several important limitations. First, SCr concentrations are affected by non-renal factors such as age, gender, diet, and muscle mass, especially in young children (41). Furthermore, the concentration may not start to rise until 25% to 50% of nephron function has been lost, whereas at low levels of renal function, active tubular excretion of creatinine will overestimate actual GFR (1, 47, 48). In addition, SCr at birth largely reflects maternal creatinine levels (47). Altogether it is generally accepted that SCr is a delayed and insensitive marker of AKI, restricted to glomerular damage, which shortcoming has driven the search for novel renal injury biomarkers (32).

BIOMARKERS

The Biomarkers Definitions Working Group and the Biomarker Consortium have defined biomarkers as characteristics that can be objectively measured and evaluated as indicators of normal biological processes, pathogenic processes, or pharmacologic responses to therapeutic interventions (49, 50). Ideally a diagnostic or monitoring biomarker should add useful information and fulfill the following criteria: high specificity and sensitivity, high predictive value, and high robustness (51, 52). The biomarker validation process can be described in three successive stages: exploratory, probable valid, and known valid stage (53).

GFR biomarkers

Serum Cystatin C

Cystatin C (CysC) is a 13 kDa protein that is produced at a constant rate by all nucleated cells and is freely filtered by the glomerulus, reabsorbed and catabolized by the proximal tubules. It is not excreted in other parts of the kidneys (54, 55). Importantly, CysC is thought to be less affected by non-renal factors that impact creatinine and is therefore considered a better indicator of renal function, especially in individuals with a mildly reduced GFR, in whom a rise in SCr is typically not observed (so-called creatinine-blind range) (56-60). Nevertheless, recent studies found that CysC levels may be affected by other factors including increasing age, inflammatory processes, use of corticosteroids and thyroid hormone (61, 62). Evidence for the usefulness of CysC in the early detection of AKI in clinical practice is conflicting. Some studies reported a good discriminatory

function for CysC in the early detection of AKI whereas the ability to predict AKI was poor to moderate (61). A recent study by Lagos-Arevalo and colleagues evaluated daily CysC and SCr in 160 non-cardiac children admitted to the ICU (63). They found that CysC determined early after ICU admission, predicted SCr-based AKI development (AUC 0.70, 95% CI 0.53–0.89) (63). Conversely, there was no difference between CysC-AKI versus SCr-AKI in the association with clinical outcomes. When summarizing, although their findings did not support replacing SCr with CysC to define AKI, they did not evaluate "true CysC baseline values" obtained prior to ICU admission, which might have influenced the performance of CysC (63).

Serum β-Trace protein

β-trace protein (BTP) is a low-molecular mass glycoprotein (23-29 kDa depending on the degree of glycosylation) that is generated at a constant rate by glial cells in the central nervous system (64, 65). Like CysC, BTP is freely filtered by the glomerulus and reabsorbed by the proximal tubule with minimal non-renal elimination (66). Several studies demonstrated a good correlation between serum BTP concentrations and GFR measurements, suggesting it as a potential alternative to SCr (67, 68). Filler and colleagues even showed that BTP was superior to SCr and a good alternative for CysC in the detection of mildly reduced GFR in children (68). Treatment with glucocorticoids results in dose-dependent overestimation of GFR by calculations based on BTP (62), as had been shown for CysC. The assumption that, in contrast to SCr, neither CysC nor BTP crosses the placental barrier has recently been questioned (69-73). In fact, it was suggested that at least some CysC may cross the placenta whereas BTP may not, which makes BTP of particular interest for estimating GFR in newborns (70). Currently, the performance of BTP as a marker of GFR in critically ill children is unknown. Finally, in addition to its role as a biomarker of renal function, BTP has lately emerged as a biomarker of cardiovascular risk, which may interfere with its function as a marker for kidney injury in ICU patients (74).

Tubular injury biomarkers

Neutrophil gelatinase-associated lipocalin

Human neutrophil gelatinase-associated lipocalin (NGAL) is a small 25 kDa protein, covalently bound to neutrophil gelatinase, and originally discovered in activated human neutrophils (75, 76). NGAL is expressed at a low level in other human tissues including the lungs, small intestine, liver, heart and healthy kidneys, where it inhibits bacterial growth by interference with siderophore-mediated iron acquisition, and induces epithelial cell growth (77-79). Upon kidney injury, NGAL reabsorption by the proximal tubule may be diminished whereas de novo synthesis in the distal tubules can be strongly upregulated.

Especially the latter mechanism contributes to a significant increase in urinary NGAL excretion (80).

In a landmark paper, Mishra and colleagues were the first to evaluate NGAL as an early biomarker of AKI among 71 children who underwent cardio-pulmonary bypass (CPB); 28% children developed AKI defined as a 50% increase in SCr. Using SCr, AKI was not diagnosed until 1-3 days after CPB, whereas urine and serum NGAL concentrations rose ten-fold or more within 2 hours after CPB. The area under the ROC curve to predict SCr based AKI at 2 hours after bypass was 0.906 for serum NGAL and 0.998 for urine NGAL. Moreover, urine NGAL level 2 hours after CPB was independently associated with the duration of AKI (81). Subsequent studies, mainly in adults, evaluated uNGAL across several clinical settings, including critical care and kidney transplantation, and confirmed this finding (82, 83). Studies then focussed on larger populations and evaluated NGAL for predicting length of hospital stay, morbidity, and mortality (84-86). Data on the performance of NGAL in a heterogeneous pediatric critical care setting are scarce as none of the studies provided insight in the biomarker evolution using time-intervals based on hours shortly after ICU admission (87-91). Besides, studies were limited in that sample sizes were small and age ranges were wide, from one week to 21 years (87-91)

Kidney injury molecule-1

Kidney injury molecule 1 (KIM-1) is a type 1 transmembrane glycoprotein that contains both an immunoglobulin-like and a mucin domain in its extracellular portion (92). It is expressed at very low levels in healthy proximal tubule cells and thought to promote apoptotic and necrotic cell clearance (93). After ischemic or toxic kidney injury, KIM-1 is highly upregulated and the ectodomain is shed from cells *in vitro* as well as *in vivo* into the urine and extracellular space (92-94). Similar to urinary NGAL, urinary KIM-1 was also studied for the first time in children undergoing CPB. KIM-1 was found to rise later in AKI, with 74% sensitivity and 90% specificity for prediction AKI at 12 hours post-CPB (95). In the setting of pediatric critical care, KIM-1 was solely evaluated among a limited number of asphyxiated neonates (88). Although researchers found KIM-1 to rise significantly throughout the first three days of life, the area under the curve for predicting AKI was only 0.583 (88).

Interleukin-18

Interleukin-18 (IL-18) is a pro-inflammatory cytokine that in kidney injury is induced and cleaved by caspase-1 in the proximal tubule cell (96). Cleavage converts Pro-IL-18 (24 kDa) into the mature active form of IL-18 (18 kDa), which then exits the cell resulting in the detection of IL-18 in the urine (97, 98). The first biomarker study evaluating the use of urine IL-18 (uIL-18) as an early AKI biomarker in critically ill patients concerned adults, whose uIL-18 rose 24h before SCr did in case of AKI (99). To substantiate this finding in

critically ill children, the Cincinnati group performed a prospective study using the same CPB cohort as was used for the NGAL studies (81, 100). Parikh and colleagues demonstrated that uIL-18 concentrations were indeed increased at 4 to 6 hours after CPB in children who suffered from AKI. At 12 hours uIL-18 peaked over 25-fold compared to pre-surgery levels and remained elevated for the next 24 to 48 hours. The area under the receiver operating curve (ROC) for uIL-18 at 4, 12 and 24 hours post-CPB were 0.61, 0.75, and 0.73, respectively (100). In contrast, Lui and colleagues observed that IL-18 levels measured in serum rather than urine did not differ between children with and without AKI (101). In a cohort of mechanically ventilated children it was shown that peak uIL-18 levels increased with worsening of the maximal pRIFLE strata achieved; statistically significant differences became apparent only upon the day of AKI onset (102). Thus, the use of IL-18 in the PICU setting may be challenging given its role during inflammation thereby introducing the possibility of disease-specific alterations.

L-type fatty acid-binding proteins

Human intracellular fatty acid-binding proteins (FABP) are expressed from a large multigene family and encode 14 kDa proteins that are members of the superfamily of lipid-binding proteins (LBP) (103). There are nine different FABP with tissue-specific distribution that includes liver, intestinal, muscle and heart, adipocyte, epidermal, ileal, brain, myelin, and testis (104). In the human kidney, L-FABP is predominantly found in the cytoplasm of renal proximal tubules (105). During injury from hypoxia, L-FABP binds fatty acid oxidation products and limits the toxic effects of oxidative intermediates on cellular damage. Transporting these oxidative products to the mitochondria or peroxisomes, where they will be oxidized, L-FABP is involved in intracellular fatty-acid homeostasis (106). Another study demonstrated *in vitro* that L-FABP is an important cellular antioxidant during oxidative stress induced by hydrogen peroxide and hypoxia-reoxygenation (107).

At the first clinical evaluation, urinary L-FABP was examined in children undergoing CPB (108). Urinary L-FABP in the AKI patients measured 4 hours after the surgery was significantly higher than in non-AKI patients, whereas SCr started to increase after 24–48 h in the AKI patients. ROC curve analysis for post-CPB AKI diagnosis revealed an area under the ROC curve of urinary L-FABP (4 hours after surgery) of 0.810 (108). Krawczeski and colleagues investigated the temporal pattern and predictive value of four biomarkers including L-FABP. L-FABP increased at 6 hours post-CPB and was demonstrated to significantly improve the AUC compared to a clinical model (86). These findings were confirmed in subsequent studies, both in adults and children in various clinical settings (109-111).

Growth factors and transcription factors

Soler Garcia and colleagues identified a novel urinary biomarker profile to monitor the outcome of children with HIV-associated nephropathy (HIVAN) (112, 113). Children with HIVAN show glomerular and tubular changes, including mesangial hyperplasia leading to glomerulosclerosis with proteinuria, microcystic changes of proximal tubular cells, as well as necrosis and sloughing of renal tubular epithelial cells into the lumen. In correlation with the development of these lesions, the urinary levels of the angiogenic growth factor fibroblast growth factor 2 (FGF-2) and the matrix metalloproteinase 2 (MMP-2) were significantly elevated in these patients. In addition, it was demonstrated that the urinary concentration of epidermal growth factor, (EGF), a powerful growth factor for renal epithelial cells, was decreased in children with HIVAN (112-114). Seeing that many of the renal histological lesions in children with HIVAN can mimic the renal lesions in critically ill children with AKI secondary to septic shock, post-cardiopulmonary bypass or ECMO, these new biomarkers could be a valuable addition to e.g., NGAL and KIM-1.

The group from Washington published two pilot studies on urinary NGAL (uNGAL) in critically ill children (91, 115). One study showed that in the early stages of a critical illness the urinary levels of NGAL and FGF-2 were sensitive, but not specific, to identify critically ill neonates at risk of AKI. Low EGF levels post-recovery identified critically ill neonates with AKI (91). The other study showed that a biomarker profile comprised of uNGAL, uFGF-2, and uEGF increased the specificity to detect AKI in critically ill children, when compared to each biomarker used alone. Also, uNGAL and uFGF-2 may predict the risk of death. Still, further validation of these findings in a large sample size is warranted (115).

CONCLUSIONS

To summarize; AKI is a frequent complication in children admitted to an ICU and is associated with significant short-term morbidity and mortality. Tubule-interstitial pathological changes caused by AKI may result in residual CKD on the long term. Currently, the diagnostic paradigm for AKI relies largely on traditional parameters of GFR including SCr and UO. SCr typically lacks sensitivity for detecting AKI as it is a measure of glomerular function rather than tubular damage, and it is affected by several non-renal factors. This important limitation has driven the search for new biomarkers for measuring GFR, including CysC and BTP, as well as for detecting tubular injury and recovery, such as IL-18, L-FABP, NGAL, KIM-1, FGF-2, EGF, and MMP-2.

Aims and outline of this thesis

The aims of this thesis are:

- To assess reference values of a new set of biomarkers of kidney function in infants without kidney injury
- To evaluate the incidence and severity of AKI in critically ill infants, grouped by treatment with or without ECMO, using RIFLE-criteria as well as urinary biomarkers
- To determine the prevalence and predictive factors of CKD during long-term follow-up of children previously treated with neonatal ECMO

Part II will focus on reference values of new AKI biomarkers. In **chapter 2 and 3** reference values of glomerular filtration biomarker BTP, and tubular injury biomarkers NGAL and kidney injury molecule-1 KIM-1 were established in children born term (at least 37 weeks of gestation) aged one day to one year.

Part III focusses on the incidence, etiology, and diagnosis of AKI, addressing the value of new urinary biomarkers NGAL and KIM-1. In **chapter 4** a cohort study was performed to systematically evaluate the incidence and clinical course of AKI in critically ill neonates receiving ECMO support. In **chapter 5** we evaluated risk factors associated with AKI following ECMO decannulation in children who also received pre-emptive continuous hemofiltration (CH) as standard of care. In **chapter 6** a prospective observational trial was conducted to characterize temporary urinary NGAL and KIM-1 patterns following ICU admission in critically ill children up to one year of age. The patterns of these urinary biomarkers were also evaluated in children prior to- and during ECMO support and concomitant CH in **chapter 7**. **Chapter 8** evaluated urinary biomarkers associated with renal recovery in a subgroup of critically ill children.

Part IV describes a cross-sectional study, performed to determine the prevalence of CKD and evaluate its predictive factors during long-term follow-up of children and adolescents previously treated with neonatal ECMO (**chapter 9**).

Part V summarizes the results of these studies, puts them in perspective and speculations on areas of current and future research topics (**chapters 10 and 11**).

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PART II

REFERENCE VALUES

2 Reference ranges for serum β-trace protein in neonates and children younger than 1 year of age

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ABSTRACT

Background: β-Trace protein (BTP) has been proposed as an alternative endogenous marker of glomerular filtration rate. Data on BTP reference ranges in young children are scarce. We therefore aim to establish reference ranges and examine the developmental course of serum BTP in basically healthy children younger than 1 year of age.

Methods: Single blood samples were taken from healthy children (born at gestational age ≥37 weeks) <12 months of age. Serum BTP was measured using the N latex B-trace protein assay Siemens Diagnostics, Deerfield, IL, USA) on an Immage® 800 Rate Nephelometer (Beckman Coulter Inc. Brea, CA, USA). Serum creatinine and cystatin C were additionally determined and compared to reference values to confirm a normal renal function.

Results: From June 2010 to January 2014, 95 blood samples were collected from 95 children (67.4% male; median age 120 days (inter quartile range 57 – 166)). BTP was normally distributed (mean concentration 0.84 ±standard deviation 0.35 mg/L). Considering all children, the 50th centile BTP reference concentration was 0.82 mg/L (5th – 95th centiles; 0.27 – 1.38). BTP concentrations were the highest in neonates and steadily declined with increasing age (Spearman's rank correlation was -0.415, *P*=0.002). No gender differences were found.

Conclusions: Our data provide a BTP reference range for the first year of life. Seeing the biological pattern of BTP, with only a limited postnatal decline, this marker might offer a promising alternative to serum creatinine-based methods for estimating glomerular filtration rate in newborns.

INTRODUCTION

Accurate assessment of renal function in children is important in a number of clinical situations including screening and/or monitoring of renal disease. Renal function is typically measured by glomerular filtration rate (GFR), which can be directly determined from urinary or plasma clearance of an exogenous filtration marker that is biologically inert and exclusively cleared by glomerular filtration (1-3). The exogenous marker inulin is widely considered the gold standard in this respect, although its measurement is cumbersome and invasive and therefore not suitable for regular monitoring of kidney function in the clinical setting (4). An alternative is the use of endogenous markers, of which serum creatinine (SCr) is the most commonly used (4-6).

Creatinine has a molecular mass of 113 Da and is derived from creatine metabolism (5). It is not bound to plasma protein and therefore freely filtered by the glomerulus (5). However, estimating renal function using SCr has its limitations. Firstly, SCr concentrations are affected by several non-renal factors such as age, gender, diet, and muscle mass, especially in young children (7). Second, at low levels of renal function, tubular creatinine secretion will contribute to a larger extent to creatinine clearance, which results in an overestimation of actual GFR (5). Third, in neonates' SCr at birth largely reflects maternal creatinine levels (8). To overcome these limitations, alternative endogenous markers of GFR have been proposed including cystatin C (CysC) and β-trace protein (BTP).

CysC is a 13 kDa protein that is produced at a constant rate by all nucleated cells and is freely filtered by the glomerulus, reabsorbed and catabolized by the proximal tubuli. It is not excreted in other parts of the kidneys (9, 10). CysC appears superior to SCr for the detection of impaired GFR, especially in individuals with a mildly reduced GFR, in whom a rise in SCr is typically not observed (so-called creatinine-blind range) (11-13). To date, fewer studies have focused on BTP, a 23 – 29 kDa protein that is mainly produced by glial cells in the central nervous system (14). Like CysC, BTP is freely filtered by the glomerulus and then reabsorbed in the proximal tubule (15). Several studies demonstrated a good correlation between serum BTP concentrations and GFR measurements, suggesting it as a potential alternative to SCr (16, 17). Filler and colleagues even showed that BTP was superior to SCr and a good alternative for CysC in the detection of mildly reduced GFR in children (17). Finally, the assumption that in contrast to SCr neither CysC nor BTP cross the placental barrier has recently been questioned (18-22). In fact, it was suggested that at least some CysC may cross the placenta whereas BTP may not (19). This characteristic makes BTP of particular interest for estimating GFR in newborns.

Studies on the developmental course of serum BTP in young children are scarce. One study on reference ranges of serum BTP aimed to enroll subjects aged between 1 day and 21 years. Although BTP concentrations were measured in all subjects, the 10

children younger than 2 years were excluded from the reference group due to insufficient data (23). Of note, specifically the younger ones had maximal BTP concentrations whereas BTP reached a plateau at age 2 years suggesting an effect of renal maturation (23). A decrease in BTP levels directly after birth was also observed in preterm and term neonates (24). Clearly, a knowledge gap remains on the developmental course of BTP throughout the first year of life. The developmental pattern of renal functional maturation underlines the importance of filling this gap (8). The aim of our study is, therefore, to establish reference ranges and examine the developmental course of serum BTP in children younger than 1 year of age. In addition, we evaluated correlations of BTP concentrations and CysC concentrations with SCr levels, respectively.

MATERIALS AND METHODS

Setting

This was a multi-center prospective observational study conducted in the Erasmus Medical Center – Sophia Children's Hospital, Rotterdam, The Netherlands and the Albert Schweitzer Hospital, Dordrecht, The Netherlands. The study was performed from June 2010 to January 2014. The study protocol was approved by the Erasmus MC University Medical Center Institutional Review Board. We obtained written informed consents from parents or legal guardians of all infants prior to the study start.

Basically healthy full-term children (at least 37 weeks of corrected gestational age at the time of entry in the study) were randomly selected from those admitted to the general pediatric ward or pediatric surgical ward of one of the two hospitals. Health status was determined on the basis of medical history and physical examination by the attending physician prior to the study start to exclude illnesses including bacterial or viral infections. Also, children were not eligible for inclusion when they had: 1) congenital abnormalities of the kidney or urinary tract; 2) suffered from prerenal problems before or during hospital admission (e.g., dehydration, shock, sepsis); 3) had skeletal muscle disease; 4) previously or currently received nephrotoxic drugs (e.g., vancomycin, gentamycin or cytostatic drugs) or glucocorticoid medication. Finally, children were excluded from the data analysis when the blood sample volume (<0.5 mL) was insufficient to measure all filtration markers (SCr, CysC and BTP). Finally, the following demographic data were collected for each subject: gender, ethnicity (Caucasian yes or no, based on parent identification), age, weight, and admission diagnosis at the time of sampling.

Sample collection and processing

Blood samples were obtained by either capillary or venous puncture once following hospital admission but prior to elective surgery or any other medical procedure, if

applicable. Each blood sample was centrifuged within 30 minutes after blood draw, aliquoted and stored at -80ºC until measurement of SCr, CysC and BTP. All filtration marker measurements were performed in the hospital's routine clinical laboratory. SCr was measured by an enzymatic assay (Creatinine Plus; Roche Diagnostics, Banchburg, NJ, USA) on a Cobas 8000-702 analyzer (Roche Diagnostics, Almere, The Netherlands). Serum CysC was measured using a particle-enhanced immunoturbidimetric assay (Tina-quant®; Roche Diagnostics, Indianapolis, IN, USA) on a Cobas 8000-502 analyzer (Roche Diagnostics, Almere, The Netherlands). Serum BTP was measured using the N latex β-trace protein assay (Siemens Diagnostics, Deerfield, IL, USA) on an Immage 800 Rate Nephelometer (Immage® 800, Beckman Coulter Inc. Brea, CA, USA). The BTP assay range was 0.25 – 15.8 mg/L with an intra- and inter-assay coefficients of variation of 4.2% and 11.9%, respectively.

Statistical analysis

Continuous data are presented as mean values with standard deviation (SD) or median values with inter quartile range (IQR). Categorical variables are presented as numbers with percentages (%). To confirm a healthy renal status in all subjects, measured SCr concentrations were compared to previously published age-specific SCr reference values and converted into Z-scores using the formula: Z-score = (SCr subject – Y)/standard deviation (0.0845). Y denotes the ^{10}log [creat(µmol/L)] and was estimated for each individual subject as described in more detail elsewhere (25). Z-scores below −2 or higher than 2 were considered abnormal.

Based on the physiological postnatal maturation of renal function, the following age-groups were distinguished: day 1 – week 1, week 2 – 1 month, month 2, month 3, months 4 – 6, month 7 – 12. Parametric reference values (5^{th}, 10^{th}, 20^{th}, 50^{th}, 80^{th}, 90^{th}, 95^{th}) were then calculated for each age according to the Generalized Additive Model for Location, Scale and Shape (GAMLSS) method (26). In this approach many distributions can be fitted on the data assuming that the mean, coefficient of variation and (possibly) skewness and kurtosis are smooth functions of age. We used the Akaike Information Criterion (AIC) to decide which of the models is best. This criteria balances a good model fit with parsimony (27). In the fitted models we considered that the BTP has a detection limit of 0.25, so it was left censored. Correlation between BTP and CysC with SCr was evaluated with the Spearman's rank correlation coefficient test. Two-sided P=0.05 was considered the limit of significance in all analyses. Statistical analysis was performed using IBM SPSS statistics version 20 (Statistical Package for the Social Sciences, Chicago, IL, USA) and R 3.0.1 (R Core Team 2013).

RESULTS

Patients

In total 112 children were enrolled in the study. Seventeen (15%) were excluded because the volume of blood sampled was too small. Thus, the reference range was calculated from data of 95 subjects (n=64; 67.4% male). Table 1 shows detailed patient characteristics. The median age and body weight of all 95 subjects at the time of sampling were 120 days (IQR 57 – 166) and 6.4 kg (IQR 4.1 – 7.0). Most of the subjects (n=76; 80%) were scheduled for minor surgical procedures including inguinal hernia repair (n=43) and orthopedic surgery, e.g., clubfoot repair (n=14). All calculated SCr Z-scores were normal (between −2 and 2, mean SCr Z-sc(re was −0.03 (SD ±0)95)).

Table 1. Patient demographic data and clinical characteristics for all subjects.
Continuous data are expressed as median [interquartile range (IQR)] or mean (standard deviation (SD)) and categorical data are expressed as number (%).

Patient characteristics	All patients (n=95)
Gender, *male, n (%)*	64 (67.4)
Ethnicity, *Caucasian, n (%)*	82 (86.3)
Age at sampling, *days, median (IQR)*	120 (57 – 166)
Weight at sampling, *kilograms, median (IQR)*	6.4 (4.1 – 7.0)
Admission diagnosis, *n (%)*	
Inguinal hernia repair	*43 (45.3)*
Orthopedic surgery	*14 (14.7)*
Bronchoscopy	*12 (12.6)*
Hyperbilirubinemia	*8 (8.4)*
Sleep apnea test	*5 (5.3)*
Other	*13 (13.7)*
Serum creatinine Z-score, *mean (SD)*	-0.03 (0.95)

Considering all 95 subjects, the mean and median concentrations of BTP were 0.84 ±0.35 mg/L and 0.83 mg/L (IQR 0.56 – 1.07), respectively. The mean and median concentrations of CysC were 1.32 ±0.29 mg/L and 1.31 mg/L (IQR 1.10 – 1.48), respectively. Figure 1 is a scatter plot of both BTP and CysC concentrations versus age at sampling in days, according to gender. The mean BTP concentrations did not significantly differ between males and females: 0.86 ±0.35 mg/L versus 0.80 ±0.36 mg/L (two tailed t-test, P=0.448). BTP concentrations were the highest in the younger subjects and steadily declined with increasing age (ρ=−0.415, P=0.002 by Spearman's rank correlation analysis). CysC concentrations were also higher in younger subjects and decreased with increasing age (ρ=−0.641, P<0.001 by Spearman's rank correlation analysis).

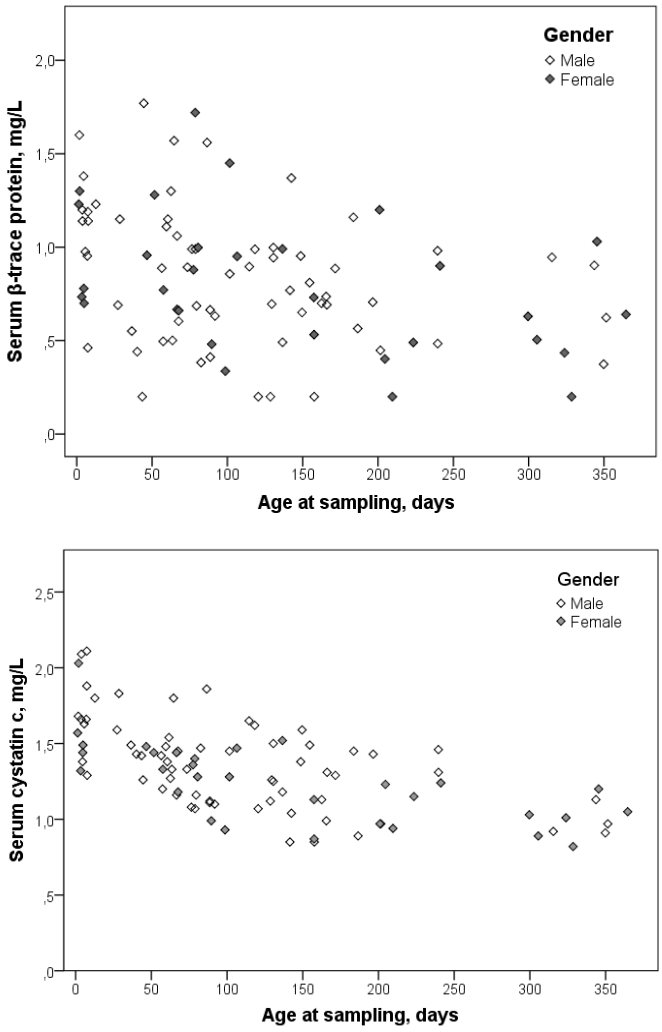

Figure 1. Serum β-trace protein (mg/L) (top) and serum cystatin C (mg/L) concentrations (bottom) versus age at sampling (days), according to gender.
The filled diamond represent females, the open diamonds represent males.

Of all the models we found that a simple normal model with constant variance was best. The mean BTP is modelled as a spine of the logarithm of age. Not too much flexibility is needed, as the fitted function is almost linear. In fact, the difference in the AIC between the best model and the model in which the mean BTP was a linear function of age was very small (about 0.04). Figure 2 shows the centile curves (5^{th}, 10^{th}, 20^{th}, 50^{th}, 80^{th}, 90^{th}, 95^{th}) of BTP concentrations as a linear function of age.

For all children as a group taken together the 50^{th} centile BTP concentration was 0.82 (5^{th} centile 0.27 and 95^{th} centile 1.38). BTP reference ranges (50^{th} centile with 5^{th} and

95th centile ranges) for all children as well as for the different age groups are shown in Table 2.

Subjects' SCr correlated weak but positively with BTP (ρ=0.208, P=0.044 by Spearman's rank correlation analysis) and strong and positively with CysC (ρ=0.605, P<0.001 by Spearman's rank correlation analysis) (Figure 3). Serum BTP concentrations were significantly correlated with serum CysC concentrations (ρ=0.500, P<0.001 by Spearman's rank correlation analysis).

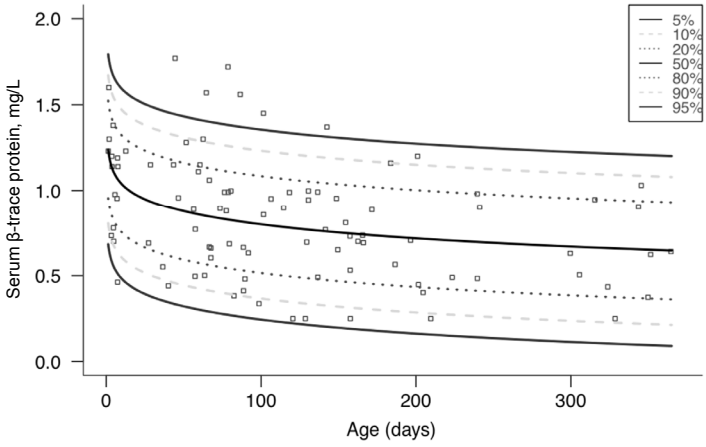

Figure 2. Reference centile curves (5th, 10th, 20th, 50th, 80th, 90th, 95th) for β-trace protein (mg/L) as a linear function of age (days). The dense black line represents the 50th centile.

Table 2. β-trace protein reference intervals, 50th centile with 5th and 95th centile ranges, for all subjects as well as stratified per age group.

		β-trace protein, mg/L		
	Number of subjects, *n*	**50th centile**	**Lower limit (5th centile)**	**Upper limit (95th centile)**
Day 1 – week 1	*11*	1.15	0.60	1.71
Week 2 – 1 Month	*6*	0.98	0.43	1.54
Month 2	*11*	0.85	0.30	1.39
Month 3	*21*	0.77	0.21	1.32
Month 4 – 6	*26*	0.70	0.15	1.26
Month 7 – 12	*20*	0.67	0.11	1.22
All subjects	*95*	0.82	0.27	1.38

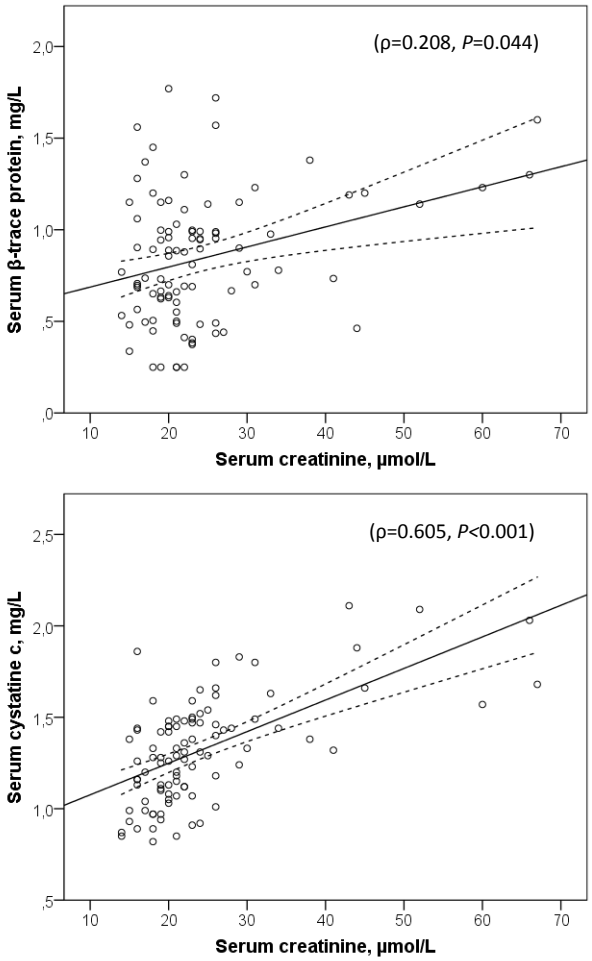

Figure 3. Correlation between, respectively, serum β-trace protein (mg/L) (top) and serum cystatin C (mg/L) concentrations (bottom) with serum creatinine concentrations (µmol/L).
Correlation between BTP and CysC with SCr was evaluated with the Spearman's rank correlation coefficient test. The dense black line represents the mean and the dotted lines the 95% confidence intervals. BTP, β-trace protein; CysC, Cystatin C; SCr, Serum Creatinine.

DISCUSSION

To the best of our knowledge data on reference ranges for the levels of serum BTP in young children are scarce. This study presents reference ranges for serum BTP in children up to 1 year of age and shows that BTP concentration peaks at birth and then declines with increasing age. Strict inclusion criteria were applied to ensure that the study cohort consisted of children with normal renal function. Furthermore, concentrations of SCr and CysC, both well-characterized markers of GFR for this age group, were also measured in

all blood samples (11, 13, 28). The SCr Z-scores were all in the normal range (>−2 and <2). Moreover, CysC concentrations were in perfect agreement with previously published reference intervals measured by the immunoturbidimetric method by Harmoinen and colleagues (younger children 0.75 – 1.87 mg/L) (29). Hence, both SCr and CysC confirm that our reference population was adequate for the purpose as all enrolled patients had a healthy renal status.

The values established in this study will help fill an important gap in the clinical laboratory reference values for BTP. Previous studies either focused on pre-terms and neonates alone, or on 2 – 21 year olds, while it is known that GFR evolves considerably during the first year of life (5). Bariciak and colleagues found that median BTP levels decreased from 1.85 mg/L to 1.27 mg/L from the first to the third day of life in neonates ≥36 weeks of gestational age (24). The levels established in our study are slightly lower than those of Bariciak and colleagues. One possible explanation is that our population was ≥37 weeks of gestational age. Moreover, measurements in neonates were not performed on the first day of life, and in only three out of 17 neonates measurements were performed on day 2. The lower BTP levels therefore most likely reflect more advanced structural and functional maturation of the kidney (8). For children between the age of 2 and 21 years, BTP reference ranges reported by Bökenkamp and colleagues (mean BTP 0.69 ±SD 0.146 mg/L) were considerably lower (23). In this study, BTP concentrations had been measured in 10 children <2 years and were found to decline throughout the first 2 years of life. BTP levels measured in healthy adults are even lower (mean 0.56 mg/L; 2.5% – 97.5%, 0.39 – 0.76 mg/L) than in older children (30). Thus, the physiological course of BTP found in our study is in accordance with previous findings and strongly suggests an effect of renal maturation.

The correlation between BTP and SCr levels was weak but significant. One explanation might be that BTP in principle does not cross the placental barrier (22). As a result BTP concentrations at birth exclusively reflect the neonate's GFR whereas SCr levels in the first few days of life mainly reflect maternal kidney function. CysC was relatively strongly correlated with SCr, which, in turn, strengthens the suggestion that placental handling of both markers, BTP and CysC, might be different (19). In this context, one could also envision that BTP and CysC have different pharmacokinetic properties. Sjöström and colleagues as well as Slort and colleagues presented pharmacokinetic models in which increased non-renal elimination of CysC at a lower GFR attenuated the rises in serum CysC concentrations (31, 32). Since newborns have a lower GFR, this mechanism may possibly contribute to a more pronounced decline in CysC levels throughout the first few days of life than in BTP levels. This might, at least in part, explain the closer correlation between CysC and SCr. This explanation is supported by the findings of Olsson and colleagues who demonstrated that non-renal elimination of BTP is insignificant (33). Another theory supporting the differences in elimination patterns is the greater perme-

ability of the glomerular filtration barrier in neonates, facilitating filtration of smaller molecules (e.g., SCr, CysC) more easily than larger molecules (e.g., BTP) (34, 35).

Several limitations of this study should be recognized. First, GFR was not evaluated with a gold standard (such as inulin clearance) (4, 6). This invasive and time-consuming method would not have been approved by the Ethics Board, as in The Netherlands non-therapeutic studies in minors are only allowed by law when risks and burdens are minimal. Second, the variance in BTP concentrations was wider than that in CysC, especially in the younger ones. Although the normal distribution around the mean suggests that the current sample size should provide enough precision to reliably calculate reference ranges, one must realize that our numbers are relatively small. Particularly in view of the rapid recruitment of nephrons in the first few weeks of life that results in more pronounced and almost daily changes in BTP concentrations when compared to children beyond 1 month of age. In this light international collaboration would be useful to accumulate larger databases for the generation and validation of Box-Cox Transformations with L, M, S variables for the calculation of age-independent Z-scores. Z-scores have the additional benefit over percentiles that they provide a quantitative measure of how far a child departs from the mean value, which is especially useful for children who fall well below or above the outermost percentiles.

Altogether our data provide reference ranges for the serum BTP levels in young children up to 1 year of age. This is of importance for further evaluation of the current and future role of BTP as an alternative marker of GFR. Seeing the biological pattern of BTP, with a limited postnatal decline in the first year of life, this marker might offer a promising and easy to use alternative to SCr-based methods for estimating GFR in newborns.

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3 Reference intervals for renal injury biomarkers neutrophil gelatinase-associated lipocalin and kidney injury molecule-1 in young infants

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ABSTRACT

Background: Reliable reference intervals for two novel urinary biomarkers of renal injury, neutrophil gelatinase-associated lipocalin (uNGAL) and kidney injury molecule-1 (uKIM-1) are lacking for infants. Therefore, the aim of our study was to establish reference intervals for urinary NGAL and KIM-1 absolute concentrations as well as normalized to urinary creatinine in young infants categorized in small age intervals.

Methods: From June 2010 until March 2014, serum and urine samples of 106 basically healthy infants (born from 37 to 42 weeks of gestation) aged 1 day to 1 year were collected. Blood samples were assayed for serum creatinine levels to confirm a healthy renal status. Urine samples were assayed for creatinine, uNGAL (ng/mL) and uKIM-1 (ng/mL).

Results: Two thirds of the study cohort were boys. uNGAL concentrations declined with increasing age (likelihood ratio test, P=0.001). Also, uNGAL concentrations were higher in girls (50^{th} centile uNGAL was 27.1 ng/mL) than boys (50^{th} centile uNGAL was 14.3 ng/mL) (two tailed Wald test, P<0.001). uNGAL concentrations were not related to ethnicity. uKIM-1 concentrations were extremely low in almost all 106 subjects (median uKIM-1 was 0.08; IQR 0.08 – 0.08 ng/ml) and not related with age, gender or ethnicity (all P>0.05).

Conclusions: Our data uniquely provide uNGAL and uKIM-1 reference intervals for the first year of life. Notably, only uNGAL levels decreased with increasing age and were higher in girls. These reference intervals enable future studies to evaluate the performance of both biomarkers in detecting early kidney tubular injury, particularly in the setting of critical care.

INTRODUCTION

Acute kidney injury (AKI) is a common complication in critically ill children and is associated with an increased risk of morbidity and mortality (1). In the clinical setting most clinicians diagnose AKI based on two abnormalities: an increase in serum creatinine (SCr), which is a marker of glomerular filtration rate (GFR), and the presence of oliguria. Unfortunately, SCr is an insensitive and non-specific marker for AKI because SCr levels are influenced by a significant number of non-renal factors including age, gender, and muscle mass. Also, SCr levels will not start to rise until 25% – 50% of functioning nephrons have been lost and when renal function declines, tubular SCr secretion represents an increasing proportion of SCr clearance and overestimates GFR (2, 3). Ultimately, in newborns the diagnosis AKI is even more difficult since at birth SCr predominantly reflects maternal renal function (4). This is problematic since neonates are especially susceptible to hypovolemic kidney injury due to an inadequate renal autoregulation when blood pressure falls (5). Altogether it is increasingly accepted that SCr is a poor marker of early AKI, which has driven the search for novel replacement biomarkers in urine including neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (uKIM-1) (6).

NGAL is a small 25 kDa protein initially discovered in activated human neutrophils (7). It is expressed at a low level in other human tissues including the lungs, small intestine, liver and kidneys where it is thought to inhibit bacterial growth, scavenge iron and induce epithelial cell growth (8-10). Circulating NGAL is, after glomerular filtration, reabsorbed for more than 99% by proximal tubular cells (11). Upon kidney injury, NGAL reabsorption by the proximal tubule may be diminished whereas de novo synthesis in the distal parts of the nephrons can be strongly upregulated (11). In 2005, urine NGAL (uNGAL) was first clinically validated as an early biomarker of AKI in children after cardio-pulmonary bypass (CPB) (12). In these children high uNGAL levels at 2h post-CPB predicted the onset of AKI with 100% sensitivity and 98% specificity 2 – 4 days before AKI was identified by rise in SCr (12). uKIM-1 is a 104 kDa type 1 transmembrane glycoprotein with an immunoglobulin and a mucin domain (13). It is expressed in low levels in healthy proximal tubule cells and thought to promote apoptotic and necrotic cell clearance (14). uKIM-1 is highly upregulated after kidney ischemia or toxicity and shed into the extracellular space and urine (13). uKIM-1 was also first studied in children undergoing CPB. uKIM-1 was found to rise a few hours later in AKI, with a 74% sensitivity and 90% specificity for predicting AKI at 12h post-CPB (15).

Although both AKI biomarkers either alone or in combination proved promising, normal values in healthy persons have been scarcely studied (12, 15-18). Five studies exclusively focused on adults whereas one study enrolled both adults and children (Table 1A and B) (19-24). The latter study enrolled children stratified per subgroup of 10

Table 1A. Overview of studies on reference intervals of NGAL in children and adults

ELISA, enzyme-linked immunosorbent assay kit; IQR, Interquartile range; LOD, limit of detection; NGAL, neutrophil gelatinase-associated lipocalin; N.R., not reported; SD, Standard Deviation; SE, Standard Error; uNGAL, urine neutrophil gelatinase-associated lipocalin.

First author, year	Number	Type	Age, years	Biomarker Kit	LOD (ng/mL)	Creatinine	Normalization urine NGAL with urinary creatinine (ng/mg creatinine)	Absolute urine NGAL concentrations (ng/mL)
McWilliam et al. 2014 (27)	120 UK 171 US	Children	UK mean (9.05 (SD 4.41) US mean 11.20 (SD 2.49)	Electrochemiluminescent assays (Meso Scale Discovery, MD, USA)	0.041	Spectro photometrically	UK cohort: mean 10.71 (95% CI 8.76 – 13.11) US cohort: mean 8.19 (95% CI 6.80 – 9.86)	*N.R.*
Pennemans et al. 2013 (22)	338	Adults & Children	Mean 45.8 (range 0 - 95)	NGAL kit (RD Systems Europe, Abington, UK)	*N.R.*	Kinetic Jaffé	Mean (upper limit of 95% prediction interval) for patients aged 0-10 to 80+ years Males: 16.5 (167.5) to 30.9 (307.8) Females: 32.1 (325.4) to 60.2 (598.3)	Mean (upper limit of 95% prediction interval) for patients aged 0-10 to +80 years Males 6.67 (52.97) to 26.87 (211.16) Females: 17.94 (141.8) to 23.29 (182.58)
Rybi- Szuminska et al. 2013 (26)	172	Children	Median 9.75 (range 0.2 - 17.9)	ELISA (R&D Systems, Minneapolis, MN, USA)	0.012	*N.R.*	Males: median 1.71 (range 0.01 - 33.91) Females: median 2.53 (range 0.05 - 48.97)	*N.R.*
Cangemi et al. 2013 (25)	25 / 308	Newborns / Children	Mean 6.7 (range 0.05 - 20.7)	ARCHITECT NGAL assay (Abbott Diagnostic Italia, Rome, Itally)	0.95	Jaffé	All subjects: mean 23.4 (SD 58.8)	All subjects: mean 12.7 (SD 21.3)
Cullen et al. 2012 (20)	174	Adults	range 19 - 88	Abbott ARCHITECT i1000sr system (Abbott Park, IL, USA).	10	Jaffé	All subjects 95th centile: 14.7	All subjects < 107 (95th centile)
Madsen et al. 2012 (28)	13	Children	Median 8.3 (range 3.5 – 14.5)	Bead human kidney toxicity/ injury panel 2 (Widescreen; EMD chemicals Inc. Merck, Darmstadt, Germany)	*N.R.*	Enzymatic	Median 8.1 (range 3.4 – 19.6)	*N.R.*
Wasilewska et al. 2011 (29)	25	Children	Median 5.0 (range 0.33 - 16)	ELISA (Bioporto diagnostics, Gentofte, Denmark)	0.012	Jaffé	Median 2.31 (range 0.27 - 16.27)	Median 1.64 (range 0.25 - 5.77)
Delanaye et al. 2010 (42)	20	Adults	Mean 33.3 (SD 10.3)	Automated immuno-assay from Abbott Laboratories (Abbott Park, IL) on the ARCHITECT platform	0.022	*N.R.*	First morning sample: median 0.023 (IQR 0.012 - 0.39) Second random sample: median 0.035 (IQR 0.017 - 0.073)	First morning sample: median 29.2 (IQR 15.4 - 52.8) Second random sample: median 38.5 (IQR 20.6 - 70.9)
Wasilewska et al.2010 (30)	18	Children	Mean 7.05 (SD 4.75)	ELISA (Bioporto diagnostics, Gentofte, Denmark)	0.1	Jaffé	Median 4.2 (range 0.1 – 7.4)	Median 4.2 (range 0.1 – 12.1)
Bolignano et al. 2008(19)	10	Adults	Mean 47 (SD 12)	ELISA commercial available kit (Antibody Shop, Gentofte, Denmark)	*N.R.*	*N.R.*	*N.R.*	Mean 7.3 (SD 6.1)

Table 1B. Overview of studies on reference intervals of urine KIM-1 in children and adults

ELISA, enzyme-linked immunosorbent assay kit; IQR, Interquartile range; KIM-1, kidney injury molecule-1; LOD, limit of detection; N.R. Not Reported; SD, Standard Deviation; SE, Standard Error.

First author, year	Number	Type	Age, years	Biomarker Kit	LOD (ng/mL)	Creatinine	Normalization urine KIM-1 with urinary creatinine (ng/mg creatinine)	Absolute urineKIM-1 concentrations (ng/mL)
McWilliam et al. 2014 (27)	120 UK 171 US	Children	UK mean (9.05 (SD 4.41) US mean 11.20 (SD 2.49)	Electrochemiluminescent assays (Meso Scale Discovery, MD, USA)	0.0012	Spectro photometrically	UK cohort: 0.43 (95% CI 0.37 – 0.50) US cohort: 0.18 (95% CI 0.16 – 0.20)	*N.R.*
Fahmy et al. 2013 (21)	10	Adults	Median 48 (range 29 - 58)	Microbead sandwich ELISA on Luminex® platform (Luminex, Austin, TX)	0.0044	Jaffé	Mean 0.00107 (SE 0.0001)	*N.R.*
Pennemans et al. 2013 (22)	338	Adults & Children	Mean 45.8 (range 0 - 95)	TIM-1/KIM-1/Havcr ELISA kit (RD Systems Europe, Abington, UK)	0.059	Kinetic Jaffé	Mean (upper limit of 95% prediction interval) for patients aged 0-10 to +80 years 0.71 (3.61) to 1.58 (7.88)	Mean (upper limit of 95% prediction interval) for patients aged 0-10 to 80+ years Males: mean 0.314 (1.502) to 0.987 (3.367) Females: mean 0.239 (1.249) to 0.805 (2.898)
Wasilewska et al. 2011 (29)	25	Children	Median 5.0 (range 0.33 - 16)	ELISA (USCN Life Science, Hankou, Wuhan, China)	0.07	Jaffé	Median 0.58 (range 0.15 - 3.01)	Median 0.37 (range 0.15 - 1.82)
Chaturvedi et al. 2009 (24)	19	Adults	Median 32.0 (range 22.0 - 59.0)	TIM-1/KIM-1/Havcr ELISA kit (RD Systems Europe, Abington, UK)	0.059	Jaffé	Mean 0.228 (2SD 0.188)	*N.R.*

years (22). The six pediatric studies all reported biomarker concentrations for cohorts of children widely varying in age (25-30). One study reported higher uNGAL levels in healthy newborns (n=25, age 1 – 4 days) compared to older children (n=308, mean age 80.7 months). Nevertheless, the researchers did not report uNGAL levels adjusted for creatinine, nor did they detail the course of uNGAL throughout the post-neonatal period (25). Remarkably, in other pediatric studies on biomarker reference levels, infants were not singled out as a specific subgroup, even though it is known that physiological changes in GFR are the most pronounced during the first year of life (4, 26-29). The aim of our study was, therefore, to establish reference intervals for absolute concentrations of uNGAL and uKIM-1 as well as corrected for urinary creatinine concentration in children younger than 1 year of age. Second, we aimed to evaluate the influence of age, gender, ethnicity and minor invasive procedures on the biomarker concentrations.

METHODS

Patients

This was a prospective observational study in children admitted to either Erasmus Medical Center – Sophia Children's Hospital, Rotterdam, The Netherlands, or the Albert Schweitzer Hospital, Dordrecht, The Netherlands. The study was performed from June 2010 to March 2014. The study protocol was approved by the Medical Ethical Review Board of both institutions. Written informed consent was obtained from the primary caregivers prior to study enrollment. Considered for inclusion were otherwise healthy term infants (born between 37 and 42 week of gestation) aged 1 day to 1 year who were electively hospitalized and randomly recruited from the general pediatric or pediatric surgical ward. The latter group comprised infants who underwent minor invasive procedures that were performed by pediatric otolaryngologists and general or orthopedic pediatric surgeons, a similar methodology as previously used by other centers (31). For all subjects health status was determined on the basis of the medical history and clinical examination prior to start of the study. Infants were not considered for inclusion when they underwent either surgical procedures for conditions related to the kidney or urinary tract or major surgical procedures (e.g., cardiothoracic or extensive invasive abdominal procedures). In addition, subjects were not eligible for inclusion when they had: 1) previous episodes of renal problems and/or congenital abnormalities of the kidney or urinary tract; 2) had received nephrotoxic drugs (e.g., vancomycin, aminoglycosides); 3) suffered from muscle disease; or 4) had prerenal problems at the time of sampling (e.g., dehydration, shock, heart failure). Moreover, subjects were excluded from the study cohort when the blood or urine sample volume was insufficient to measure all biomarkers (i.e., SCr, uCr, uNGAL and uKIM-1). Also, subjects with SCr levels (SCr-based age-appropriate Z-scores >2) suggesting renal impairment were excluded as well. Finally, subjects were

excluded for logistical reasons, mainly due to the scheduling of surgical procedures to operating rooms.

Sampling

In all subjects a blood sample was obtained concurrently with the first urine sample ("sample 1") prior to any medical procedure. Moreover, in those who underwent a minor invasive procedure, a second urine sample ("sample 2") was collected within 24h post-procedure. Only "sample 1" was used to establish NGAL and KIM-1 reference intervals. "Sample 2" was exclusively used to study the influence of minor invasive procedures on both biomarker levels. Blood samples were obtained by capillary or venous puncture and directly transferred to the hospital's laboratory for SCr measurement. Urine was sampled by cotton diaper inserts, adhesive urine collection bags, or the clean catch method. Urine samples were immediately cooled at 4°C for a period of 1 – 2h, aliquoted and stored within 4h after collection at -80°C until the assay.

Creatinine, NGAL, and KIM-1 quantification

All biomarker measurements were performed in the Department of Clinical Chemistry. Creatinine was determined by an enzymatic assay (Creatinine Plus; Roche Diagnostics, Banchburg, NJ, USA) on a Cobas 8000 analyzer. During the period of sample collection, the interassay coefficient of variation (CV) was <2.6%. Total imprecision for creatinine at 5.0 and 11.74 mmol/L was 0.11 and 0.23 mmol/L, respectively.

uNGAL was measured using the uNGAL immunoassay developed for a standardized clinical platform (ARCHITECT analyzer, Abbott Diagnostics Division, Abbott Laboratories, Abbott Park, IL, USA). The mean inter-assay coefficients of variation for uNGAL was 5.3% at a concentration of 19.4 ng/mL. The limit of quantification (LoQ) of NGAL was 3.0 ng/mL. Total imprecision for NGAL at 19.4 and 201.0 ng/ml was 1.25 and 6.2 ng/ml, respectively. The reagents and calibrator for the uNGAL assays were kindly supplied by Abbott Diagnostics (Abbott Laboratories).

uKIM-1 was measured using a commercially available enzyme-linked immunosorbent assay kit (ELISA) (BioAssay Works, Ijamsville, MD, USA). The mean inter-assay coefficients of variation for uKIM-1 was <14% at a concentration of 0.17 ng/mL. The LoQ of KIM-1 was 0.08 ng/mL. Total imprecision for KIM-1 at 0.25 and 0.86 ng/ml was 0.05 and 0.07 ng/ml, respectively. uNGAL and uKIM-1 concentrations were expressed in absolute value (ng/mL) and per unit of creatinine (ng/mg creatinine) to account for the influence of urinary dilution.

Variables

The following data were collected from the electronic patients' medical records: gender, ethnicity (Caucasian yes or no, based on parent identification), age and weight on ad-

mission, and primary diagnosis leading to admission. For the patients who underwent an invasive procedure, data was collected on the type of procedure and duration of mechanical ventilation during anesthesia. Children were kept *nil per os* for 4h prior to anesthesia. Perioperative care was standardized and similar for all invasive procedures.

Statistical analysis

Data were analyzed using Statistical Package for the Social Sciences (SPSS) version 21 (SPSS, Chicago, IL, USA). All data are expressed as median values with interquartile range (IQR) for continuous variables or numbers with percentages (%) for categorical variables, unless indicated otherwise. To confirm a healthy renal status in all subjects, SCr levels were compared to previously published age-specific SCr reference ranges and converted into Z-scores using the formula: Z-score=(SCr subject – Y)/Standard Deviation (0.0845) (32). Y denotes the SCr reference concentration and was estimated for each individual subject according to the "broken stick" relationship with three log-linear segments as described elsewhere (32). A Z-score below -2 or above +2 was considered abnormal.

Parametric reference values (5^{th}, 10^{th}, 20^{th}, 50^{th}, 80^{th}, 90^{th}, 95^{th}) were calculated according to the Generalized Additive Model for Location, Scale and Shape (GAMLSS) method (33). In this approach many distributions can be fitted on the data assuming that the mean, coefficient of variation and (possibly) skewness and kurtosis are smooth functions of age. We used the Akaike Information Criterion (AIC) to decide which of the models is best. This criteria balances a good model fit with parsimony (34). In the fitted models we considered that the assay for uNGAL has a LoQ of 3.0 ng/mL, and uKIM-1 of 0.08 ng/mL so it is left censored. Based on the physiological postnatal maturation of renal function, the following age-intervals were distinguished: birth – 1 month, 2 –3 months, 4 – 6 months, 7 – 12 months. Associations of biomarker concentrations with age and gender were tested with, respectively, likelihood ratio test and two-tailed Wald test. Group differences (Caucasian versus non-Caucasian) were tested with the Mann-Whitney U test.

uNGAL and uKIM-1 followed a nearly log normal distribution. To allow a parametric approach, a logarithmic transformation was applied to the biomarker values in order to approximate a Gaussian distribution, uNGAL and uKIM-1 levels in sample 1 (prior to procedure) and sample 2 (post-procedure) were compared using a two-tailed Student's t-test. Two-sided P=0.05 was considered the limit of significance in all analyses.

RESULTS

Between June 2010 and March 2014, 184 patients met the inclusion criteria and were considered for inclusion (167 in Erasmus Medical Center – Sophia Children's Hospital and 19 in Albert Schweitzer Hospital). Seventy of those could not be enrolled since parents

refused to participate (n=25) or due to logistical problems (n=43). Eight other patients were excluded from the study cohort since either blood samples (n=3) or urine samples (n=5) were not taken. Another two were excluded because a SCr Z-score >2 suggested renal impairment. Ultimately, the study cohort consisted of 106 healthy controls.

Nineteen of these healthy controls (18%) were randomly recruited from the general pediatric ward whereas all others (n=87, 82%) were recruited from the pediatric surgical ward. Within the study period 1434 infants admitted to the surgical ward were considered for inclusion and screened for their type of medical procedure. It turned out that only 270 infants underwent a minor surgical procedure not related to their kidney of urinary tract. Of these, 119 (45%) were excluded according to all exclusion criteria. Figure 1 shows a flow chart of the number of subjects enrolled throughout the study.

Table 2 summarizes detailed patient characteristics. For all subjects (71% boys, 80% Caucasian) the median age and weight on hospital admission were 88 days (IQR 60 – 159) and 4.9 kg (IQR 4.0 – 6.8), respectively. Reasons for admission to the general pediatric ward included treatment of hyperbilirubinemia (n=8) or polysomnography (n=6), which

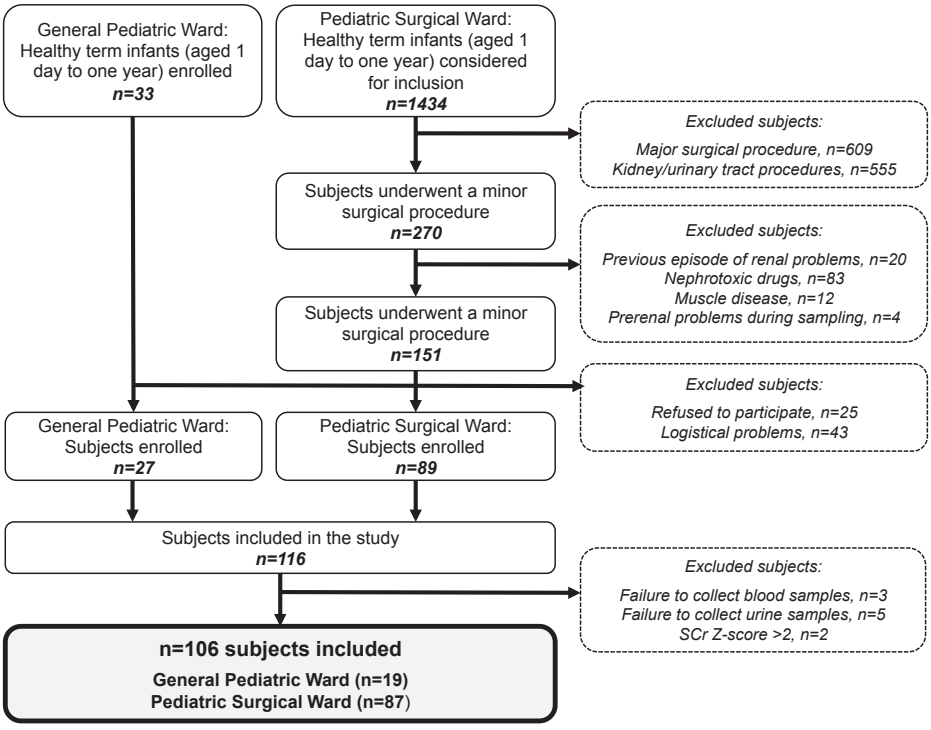

Figure 1. Flowchart of patient recruitment.
Flowchart detailing inclusion and exclusion criteria for infants aged 1 day to one year who were electively hospitalized, which resulted in the final study cohort. SCr, Serum Creatinine.

Table 2. Background and clinical characteristics for all subjects (n=106).
Continuous data are expressed as median (interquartile range (IQR)) and categorical data are expressed as number (%).

Characteristics	All subjects (n=106)
Gender, *boys, n (%)*	71 (67)
Ethnicity, *Caucasian, n (%)*	85 (80)
Postnatal age at sampling, *days, median (IQR)*	88 (60 – 159)
Weight at sampling, *kilograms, median (IQR)*	4.9 (4.0 to 6.8)
Indication for admission	
No invasive procedure, *n (%)*	19 (18)
Hyperbilirubinemia	*8 (42)*
Polysomnography	*6 (32)*
Other	*5 (26)*
Invasive procedure, *n (%)*	87 (82)
Inguinal Hernia Repair	*55 (63)*
Orthopedic surgery	*11 (13)*
Ear, nose, throat procedures	*11 (13)*
Other	*10 (11)*
Serum creatinine Z-score, *median (IQR)*	-0.11 (-0.66 – 0.63)

is routine clinical practice for infants who have experienced an apparent life-threatening event in the past and where other causes were previously excluded. Eighty-seven (82%) patients recruited from the pediatric surgical ward were admitted for an elective minor invasive procedure, such as inguinal hernia repair (n=55), orthopedic surgery (n=11), e.g., one-sided clubfoot repair, or ear, nose, throat procedures (n=11), e.g., lingual phrenectomy or nasopharyngoscopy. The median duration of mechanical ventilation during anesthesia was 51 minutes (IQR 38 – 79).

For all 106 patients the median absolute uNGAL concentration and concentration corrected for urine creatinine concentration in sample 1 (prior to any medical procedure) was 17.2 (IQR 8.3 – 37.0) ng/ml and 10.9 (IQR 5.9 – 23.2) ng/mg creatinine, respectively. Of all the models, a simple normal model with diminishing variance fitted best. The mean uNGAL is modelled as a spline based on the logarithm of age. uNGAL concentrations declined significantly with increasing age (likelihood ratio test, P=0.001). Moreover, absolute uNGAL concentrations as well as concentrations corrected for urine creatinine concentration were higher in girls than in boys (two tailed Wald test, P<0.001), even after correcting for age. Figure 2 shows the centile curves of absolute uNGAL concentrations as a linear function of age according to gender.

The 50th centile uNGAL concentration was 27.1 (5th centile 5.5 and 95th centile 136.9, Figure 2A) ng/mL and 14.3 (5th centile 2.9 and 95th centile 72.3, Figure 2B) ng/mL for all girls and boys, respectively. uNGAL reference intervals, both absolute and normalized

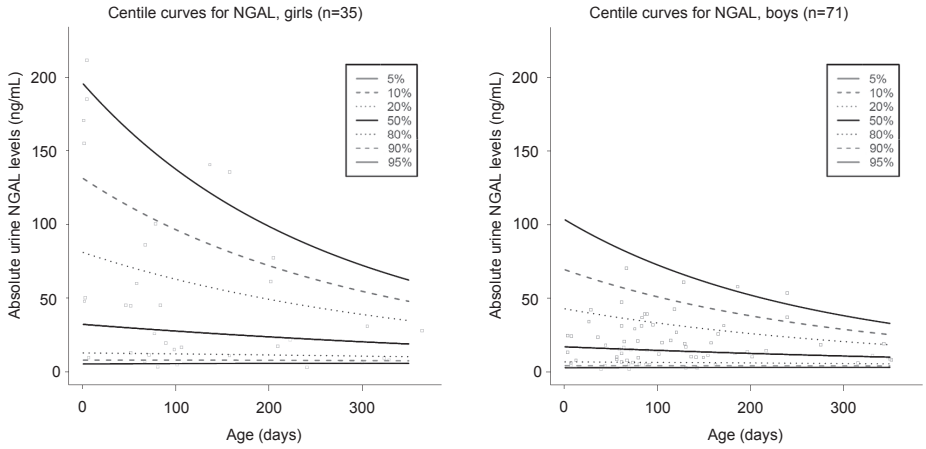

Figure 2. Reference intervals (5th, 10th, 20th, 50th, 80th, 90th, 95th centile curves) of absolute urine NGAL concentrations in young infants according to gender, either *(Figure 2A)* girls or *(Figure 2B)* boys. The dense black line represents the 50th centile.

Table 3. Reference intervals, 50th centile with 5th and 95th centile ranges, of absolute urine NGAL concentrations in four age intervals, according to gender.
NGAL, neutrophil gelatinase-associated lipocalin.

Age category	Gender, n	Urine NGAL Reference intervals					
		Absolute urine concentration (ng/mL)			Normalization with urinary creatinine (ng/mg creatinine)		
		5th Centile	50th Centile	95th Centile	5th Centile	50th Centile	95th Centile
1 Month	Boys, n=8	2.8	16.7	99.0	2.6	14.2	78.1
	Girls, n=7	5.3	31.6	187.3	5.0	27.5	151.0
2 – 3 Months	Boys, n=30	2.9	15.5	83.8	2.4	12.0	60.4
	Girls, n=12	5.5	29.4	158.5	4.7	23.3	116.8
4 – 6 Months	Boys, n=20	3.0	13.8	63.8	2.1	9.1	39.5
	Girls, n=6	5.6	26.0	120.8	4.1	17.6	76.3
7 – 12 Months	Boys, n=13	3.0	14.3	72.3	1.7	5.9	20.8
	Girls, n=10	5.7	21.4	80.1	3.2	11.4	40.3
Overall	***Boys, n=71***	***2.9***	***14.3***	***72.3***	***2.2***	***10.3***	***49.7***
	Girls, n=35	***5.5***	***27.1***	***136.9***	***4.2***	***19.9***	***96.1***

for urinary creatinine (50th centile with 5th and 95th centile ranges), are shown in Table 3, categorized for gender and age. There was no significant difference in uNGAL levels between Caucasian and non-Caucasian participants (Mann-Whitney U test, $P>0.05$).

For all 106 patients the median absolute uKIM-1 concentration in urine and concentration corrected for urine creatinine in sample 1 (prior to procedure) were 0.08 (IQR 0.08 – 0.08) ng/ml and 0.06 (IQR 0.03 – 0.10) ng/mg creatinine, respectively. Thus, in most patients uKIM-1 was below the LoQ and did not change with increasing age. Also, uKIM-1 concentrations did neither significantly differ between boys and girls nor between Caucasian and non-Caucasian participants (Mann-Whitney U tests, all $P>0.05$). Thus, it was not possible to compute uKIM-1 reference values categorized for gender and age.

A second urine sample collected within 24h post-procedure was available for 83 of the 87 subjects who underwent a minor invasive procedure (95%). The median time elapsed until collection was 15 hours (IQR 10 – 19). For these 83 subjects, none of the biomarkers concentrations measured statistically significantly differed between sample 1 (prior to procedure) and sample 2 (post-procedure) (logarithmic transformation, all $P>0.05$ by two-tailed Student's t-test).

DISCUSSION

This study presents reference intervals for uNGAL and uKIM-1 in children up to one year of age, divided in small age categories. To ensure that the participants accurately represent a healthy population with a normal renal function, strict inclusion criteria were applied, one of which was the use of age specific Z-scores of SCr.

Availability of these reference intervals will help fill a gap in clinical laboratory reference values of both urinary biomarkers. Only three of all previous studies reporting uNGAL levels for healthy subjects used the same biomarker assay (ARCHITECT NGAL assay, Abbott Diagnostics) and only one was a pediatric study. This study by Cangemi and colleagues evaluated newborns (n=25, age 1 – 4 days) and children (n=308, mean age 80.7 months) and reported significantly higher uNGAL levels for newborns (median 30.3 ng/mL) than for older children (median 4.6 ng/mL). Unfortunately, detailed uNGAL data throughout the first year of life were not provided (25). uNGAL levels found in our study (median absolute NGAL 17.2 (IQR 8.3 – 37.0) ng/ml) are in line with data reported by Cangemi: uNGAL levels in the youngest of our study group were somewhat lower than those of the younger newborns in the Cangemi study. Conversely, the levels of our study group aged >6 months were slightly higher than those of their 6-year-old children.

uNGAL levels in the present study peaked after birth, then gradually dropped and remained low towards the end of the first year of life. This is surprising since in healthy renal tissue uNGAL is normally expressed at very low levels but up regulated in response

to tubular injury. One explanation for the time-related change in uNGAL levels could be related to normal physiological renal maturation. NGAL has been shown to be involved in epithelial proliferation and embryonic renal maturation in rats (11). Furthermore, NGAL also promotes a certain proliferative effect on damaged tubular epithelial cells in adult kidneys (35). The precise molecular mechanism through which NGAL exerts its growth effects on renal cells is not yet known however (36).

The gender difference demonstrated in this study is another important finding: uNGAL levels in girls were higher than in boys. Others have reported this for young adults, children and even very low body weight neonates. Huyn and colleagues suggested that it might be due to contamination with vaginal secretion containing neutrophils (22, 27, 37, 38). In this light it would be interesting to have urine collected by an indwelling catheter, however it was considered unethical to use this method in our cohort of basically healthy infants. Nevertheless, AKI patients usually present with dramatically increased NGAL levels, up to thousands instead of hundreds ng/ml which implies that the variance due to gender seems to be less important for the diagnosis of AKI (12).

All previous studies on KIM-1 concentrations in healthy subjects used different type of assays. The assay we used was robust and suitable for research and clinical use as the performance characteristics of this ELISA were consistent over time. Using this assay we found that uKIM-1 concentrations in most patients were undetectable (below the lower limit of detection; 0.08 pg/ml), which clearly indicates that uKIM-1 concentrations in healthy children up to one year of age are extremely low. This finding suggests that uKIM-1 may serve a useful purpose in identifying early proximal tubular damage as any rise in urine concentration indicates injury. This applies to newborns in particular in whom detection of early renal dysfunction is even more problematic due to the maternal contribution to SCr level (4).

There is an ongoing debate regarding the normalization of urinary biomarker concentrations to urinary creatinine (39). As creatinine excretion is unstable in AKI, changes in biomarker/creatinine ratios cannot be correctly interpreted unless the directional change of each biomarker is known (40). In healthy normally hydrated, controls, urinary creatinine excretion is stable and does not add to the interpretation of biomarker levels (22, 27, 37, 38). This was also the case in our study. In fact, we additionally explored uNGAL levels corrected for creatinine and found a highly significant association with age and gender consistent with the analysis for absolute uNGAL levels.

Our data show that minor elective surgery does not alter uNGAL and uKIM-1 levels suggesting that tubular injury does not occur during these procedures. We must realize, however, that the timing of collection of the second sample might not have been appropriate for detecting an increase in biomarker concentration. Previous research showed that uNGAL and uKIM-1 levels, in the setting of AKI following cardiac surgery, tended to rise sequentially (17). uNGAL levels significantly increased in AKI patients at 2h after CPB

initiation and uKIM-1 levels increased at 12 hours (17). Therefore the post-procedure sample may have been collected too late as it was collected only after an average of 15h. However, if there would have been any substantial peak in biomarker concentrations, we would expect to still detect a higher level at this time.

Several limitations of this study should be addressed. First, we did not use a gold standard such as inulin clearance to measure GFR and we did not use an indwelling bladder catheter to collect urine (3, 41). Both methods are invasive procedures and were considered unethical in this study population. Second, the number of patients enrolled during the first week of life was relatively small. For this reason we were not able to establish urine uNGAL reference values stratified per day during this period. This is unfortunate taking into consideration the renal hemodynamics and functional changes throughout the perinatal episode (4). Due to these small sample sizes, there is greater uncertainty, on a relative basis, at the extreme percentiles (5^{th} centile and 95^{th} centile) than near the central percentiles. Nonetheless, this study uniquely presents data on uNGAL and KIM-1 concentrations in a substantial number of basically healthy infants. Fourth, a considerable number of patients were excluded because of logistical problems (43 out of 184, 23%) since there was only one researcher appointed for patient inclusion whereas patients were regularly scheduled for surgery at the same time. Last, the results of our study cannot be fully compared to other studies that used different biomarker assays. Even though, our uNGAL data perfectly fill the age gap in the one study that used a similar assay (25).

Altogether our data provide uNGAL reference levels in basically healthy infants for the first year of life. We demonstrated that uNGAL levels decrease throughout the first year of life and are higher in girls when compared to boys. uKIM-1 levels were below the detection limit in most subjects and did not correlate with age, gender or ethnicity. These reference intervals enable to evaluate the performance of both biomarkers in detecting early tubular injury, specifically in young children in whom the detection of AKI is even more problematic due to the maternal influence on SCr levels and age-related physiological changes in GFR.

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PART III

ACUTE KIDNEY INJURY – ETIOLOGY & BIOMARKERS

4 Acute kidney injury is a frequent complication in critically ill neonates receiving extracorporeal membrane oxygenation: a 14-year cohort study

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ABSTRACT

Introduction: Newborns in need of extracorporeal membrane oxygenation (ECMO) support are at high risk of developing acute kidney injury (AKI). AKI may occur as part of multiple organ failure and can be aggravated by exposure to components of the extracorporeal circuit. AKI necessitates adjustment of dosage of renally eliminated drugs and avoidance of nephrotoxic drugs. We aimed to define systematically the incidence and clinical course of AKI in critically ill neonates receiving ECMO support.

Methods: This study reviewed prospectively collected clinical data (including age, diagnosis, ECMO course, and serum creatinine (SCr) of all ECMO-treated neonates within our institution spanning a 14-year period. AKI was defined by using the Risk, Injury, Failure, Loss of renal function, and End-stage renal disease (RIFLE) classification. SCr data were reviewed per ECMO day and compared with age-specific SCr reference values. Accordingly, patients were assigned to RIFLE categories (Risk, Injury, or Failure as 150%, 200% or 300% of median SCr reference values). Data are presented as median and interquartile range (IQR) or number and percentage.

Results: Of 242 patients included, 179 (74%) survived. Median age at the start of ECMO therapy was 39 hours (IQR 26 – 63); median ECMO-duration was 5.8 days (IQR 3.9 –9.4). In total, 153 (64%) patients had evidence of AKI, with 72 (30%) qualifying as Risk, 55 (23%) as Injury and 26 (11%) as Failure. At the end of the study period only, 71 (46%) patients of all 153 AKI patients improved by at least one RIFLE category. With regression analysis, it was found that nitric oxide ventilation (P=0.04) and younger age at the start of ECMO (P=0.004) were significant predictors of AKI. Survival until intensive care unit discharge was significantly lower for patients in the Failure category (35%) as compared with the Non-AKI (78%), Risk (82%) and Injury category (76%), with all P<0.001, whereas no significant differences were found between the three latter RIFLE categories.

Conclusions: Two thirds of neonates receiving ECMO had AKI, with a significantly increased mortality risk for patients in the Failure category. As AKI during childhood may predispose to chronic kidney disease in adulthood, long-term monitoring of kidney function after ECMO is warranted.

INTRODUCTION

Extracorporeal membrane oxygenation (ECMO) is an advanced cardiopulmonary bypass (CPB) technique providing mechanical life support to critically ill patients with acute reversible respiratory or cardiovascular failure, not responding to conventional intensive care. Many neonatal ECMO candidates already have an inflammatory response before the start of ECMO because of asphyxia, hypoxia, infection, or shock. Unfortunately for these already critically ill patients, exposure to components of the ECMO circuit can aggravate this inflammatory response, resulting in a so-called capillary leakage syndrome (1). High levels of circulating endotoxins, exotoxins, interleukins and leukotrienes from activated leucocytes and thrombocytes as well as complement factors, bring harm to the capillary basement membranes (2). This leads not only to water and small-molecule leakage through the capillary membrane, but also to leakage of relatively large molecules, including albumin, resulting in generalized oedema. The blood pressure will decrease because of extravasation of water and proteins, necessitating administration of vasopressor drugs. Low blood pressure and tissue oedema may cause deficient tissue perfusion and oxygenation, leading to further organ failure, of which aggravation of existent lung failure and oliguric kidney failure are most prominent.

Acute kidney injury (AKI) requires adjustment of treatment in the short-term; for example, dosages of renally eliminated drugs should be adjusted, and nephrotoxic drugs must be avoided. Reported incidences of AKI among critically ill neonates admitted to a pediatric intensive care unit (ICU) vary widely, between 4.5% and 82.0% (3-8). This large range is partly explained by the lack of a standard definition of AKI. For that reason, the Acute Dialysis Quality Initiative group proposed a classification system for use in critically ill adult patients, termed the RIFLE criteria (9). The classification comprises three levels of severity – Risk, Injury, and Failure – and two outcomes – Loss of renal function and End-stage renal disease. It is based on changes in serum creatinine (SCr) levels and duration of oliguria. Subsequently, Akcan-Arikan and colleagues proposed a RIFLE classification adapted for pediatrics (pRIFLE) (5). The RIFLE criteria demonstrated clinical relevance for diagnosing AKI, classifying its severity, monitoring the progression of AKI, and predicting mortality in hospitalized adult and pediatric patients (7, 8, 10).

Previous research showed that renal failure is common both in adults and children receiving ECMO and is associated with increased mortality (3, 6, 11-13). By using the Extracorporeal Life Support Organization (ELSO) Registry, Askenazi and colleagues performed a large cross-sectional study in ECMO-treated neonates and children (14). Of the 7,941 neonates studied, 27.4% died. AKI (SCr >1.5 mg/dL) occurred more often in nonsurvivors than in survivors (19.0% versus 3.9% $P<0.0001$) and more nonsurvivors were treated with renal-replacement therapy (RRT) than were survivors (39.7% versus 16.0%, $P<0.0001$). This and earlier studies in ECMO-treated patients tend to be limited

for several reasons. First, the used upper limit for the definition of AKI (SCr >1.5 mg/dL) is debatable, as it does not necessarily indicate kidney injury. In newborns this cut-off value may include lower grades of AKI, whereas in older infants, this definition corresponds only to severe cases of the RIFLE category, Failure. Second, information is lacking on the time points when SCr levels peaked and on allocation of RRT, which may have prevented patients from ever reaching increased SCr levels. Therefore, the aim of this study was to define systematically the incidence and clinical course of AKI in critically ill neonates receiving ECMO support, by using SCr concentrations and consequent RIFLE categories in the time frame before we applied standard continuous hemofiltration (HF) (15). As a secondary objective we aimed to describe the relation between the severity of AKI and survival until ICU discharge.

METHODS

Setting

The ICU at the Erasmus MC - Sophia Children's Hospital, Rotterdam has been serving as an ECMO facility since 1992. This tertiary hospital is one of the two pediatric centers providing ECMO support in The Netherlands. Since 1992, more than 550 children (up to 18 years of age) have been treated with ECMO, presently at a rate of 30 ECMO runs annually. Two thirds of children receiving ECMO support are neonates. During the study period, the venoarterial bypass procedure was the common initial procedure. ECMO support was considered in patients with potentially reversible cardiac and/or respiratory failure unresponsive to optimal conventional therapy, including nitric oxide- and/or high-frequency oscillatory ventilation, maximal fluid resuscitation, and administration of inotropic and vasopressive drugs to maintain the mean arterial pressure within age-adjusted reference values.

Entry criteria for ECMO were a prolonged oxygenation index >25; prolonged alveolar-arterial oxygen difference >600 mm Hg; cardiorespiratory failure for more than 2 hours with pH <7.15 and PaO2 <5.3 kPa. Contraindications for ECMO were gestational age <34 weeks; <2.0 kilograms; mechanical ventilation for more than 10 days; preexisting intracranial hemorrhage; coagulopathy; and/or other severe congenital anomalies. During the study period, these criteria did not change.

Patients

In this cohort study, we reviewed data of all full-term neonates (≤28 days of age) who received ECMO support within our institution between January 1992 and January 2006. Patients were not eligible for the study if ECMO support had been combined with standard continuous HF for intravascular volume management, which is part of the

clinical protocol since 2005. In contrast, patients who received HF secondary to AKI were enrolled. Other reasons for exclusion were preexistenting structural renal anomalies. If a patient had undergone more than one ECMO run, only data related to the first run were included.

Variables

Data were retrieved from our electronic data registry and the hospital's 'Patient Data Management System' (PDMS), which stores all prospectively collected physiological parameters, laboratory results, and therapeutic modalities. Demographic variables were retrieved from the patients' medical records: age and weight at the start of ECMO, gender, primary diagnosis leading to the initiation of ECMO, Apgar scores at 1, 5, and 10 minutes after birth, perinatal asphyxia, cardiac arrest, the use of nitric oxide (NO) ventilation, and the administration of vasopressor drugs pre-ECMO. The following data on the ECMO run were retrieved: type of ECMO (for example, venoarterial or venovenous) and ECMO course (for example, ECMO duration, need for major surgery, and progressive heart failure during ECMO). In addition, we used length of ICU stay and survival until ICU discharge. With regard to survival, we distinguished between early nonsurvivors and late nonsurvivors using a cut-off point 24 hours post-ECMO.

AKI definition

SCr was assessed by enzymatic assay (Creatinine Plus; Roche Diagnostics, Branchburg, NJ, USA) on a Hitachi 912 analyzer, as described elsewhere (16). SCr was measured daily as part of standard clinical care. When SCr was not available on a day of treatment, the absent measurement was reported as a missing value. When more than one SCr measurement had been performed on a single day of treatment, the mean of these values was used. When HF was provided, SCr measurements were disregarded.

To determine the clinical course and severity of AKI, patients were assigned SCr-based RIFLE scores on each day of ECMO treatment: R (Risk for kidney injury), I (Injury to the kidney), and F (Failure of kidney function) (9). RIFLE strata L (Loss of renal function) and E (End-stage renal disease) were not scored, as the study period was restricted to the first 12 days of ECMO treatment. Because, in many cases, no pre-ECMO SCr concentrations were available, RIFLE categories Risk, Injury, and Failure were defined as, respectively, SCr above 150%, 200%, and 300%, of the median of locally collected SCr reference values, as recently published by our institution (17). These SCr reference values were obtained from children without kidney disease, by using small age intervals ranging from 1 day in the first week after birth up to 3 months at the end of the first year of age. Patients requiring continuous HF for AKI were included in the RIFLE category Failure. Ultimately, patients were grouped according to the highest RIFLE score attained while receiving ECMO. Improvement of AKI was defined as a decline in SCr corresponding to at least one

RIFLE category. Whereas inclusion of urine output in the RIFLE criteria has been demonstrated to be helpful to stage AKI in critically ill patients, we decided not to use our data on urine output. The reason for use of SCr exclusively for determining the RIFLE category was that diuresis varied widely, not only because of variation in kidney function, but, more important, because of changes in clinical guidelines for the use of diuretic drugs over the years of the study period (18, 19).

Statistical analysis

Data were analyzed by using Statistical Package for the Social Sciences (SPSS) version 17 for windows (SPSS, Chicago, IL, USA). All data are expressed as median values with interquartile range (IQR) for continuous variables or numbers with percentages for categorical variables, unless indicated otherwise. To obtain RIFLE scores, SCr values were expressed in percentages of the median of age-specific reference values. Univariate overall comparisons between groups (Kruskal-Wallis test or Pearson χ^2 test, as appropriate) were performed to detect differences between groups (that is, Non-AKI versus Risk versus Injury versus Failure). The Mann-Whitney U test was used to compare RIFLE distributions per ECMO day according to whether the patients survived. Significant predictors of AKI in univariate analyses were entered in multivariate analysis (logistic regression) to identify independent predictors.

To evaluate the relation between AKI severity and survival until ICU discharge, a logistic regression analysis was used to determine independent predictors for death. Reported are odds ratios (ORs) with 95% confidence intervals (95% CIs). The survival after ECMO decannulation until ICU discharge for each RIFLE category was additionally evaluated graphically by using a Kaplan-Meier survival plot and log-rank tests. Two-sided P=0.05 was considered the limit of significance in all analyses.

Informed consent

Because of the design of this cohort study, the need for ethics approval and informed consent was waived by the local Medical Research & Ethics Committee of Erasmus University Medical Center.

RESULTS

Patients

Spanning a 14-year period from January 1992 until January 2006, 282 neonatal patients received ECMO support. Thirty-five patients were excluded because they received routine HF to prevent excessive fluid accumulation, despite normal pre-HF SCr levels. For three patients, we were unable to retrieve adequate clinical data, and two patients

had congenital renal abnormalities on ultrasound. Hence in total, 242 ECMO-treated neonates were included in the study, of whom 179 (74%) survived to ICU discharge. Of the 63 non-survivors, 30 (48%) patients died within 24 hours following ECMO decannulation, primarily because of rebound pulmonary hypertension (Figure 1). Table 1 represents detailed patient characteristics grouped according to the highest RIFLE score attained during ECMO.

For all patients the median gestational age and weight on ICU admission were 40.0 weeks (IQR 38.3 – 40.6) and 3.3 kg (IQR 2.9 – 3.7), respectively. The most common primary diagnoses to initiate ECMO were meconium aspiration syndrome (MAS) (42% of all patients) and congenital diaphragmatic hernia (CDH) (29% of all patients). The primary diagnoses grouped as "other," included failure to wean from CPB after cardiac surgery, and concomitant *respiratory syncytial virus* and *Bordetella pertussis* infections, mostly in children with severe co-morbidities. Median age at the start of ECMO support was 38 hours (IQR 26 – 63). The median ECMO duration was 5.8 days (IQR 3.9 – 9.4), and all patients had been treated with venoarterial ECMO. One, however, was initially placed on

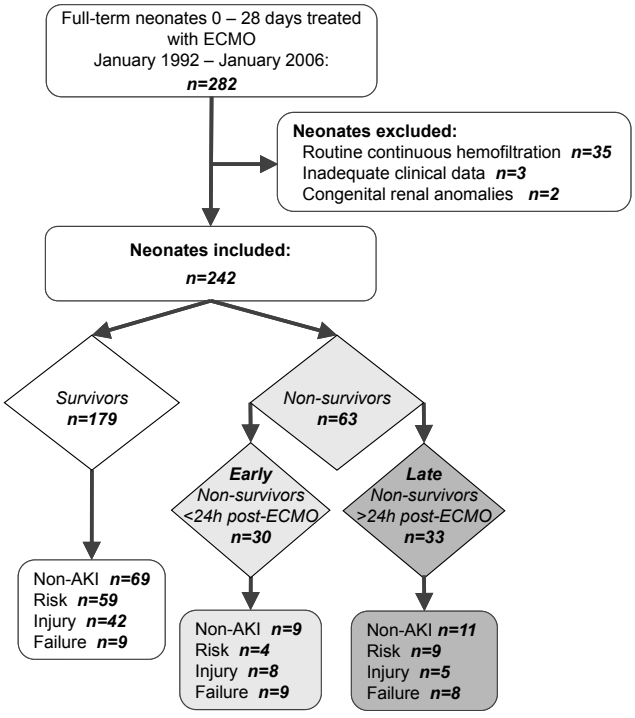

Figure 1. Patient inclusion flowchart.
Flowchart detailing inclusion and exclusion criteria for patients treated with ECMO that resulted in the final study cohort. AKI, acute kidney injury; 24h, 24 hours

Table 1. Patient characteristics grouped according to the highest RIFLE score attained
RIFLE categories Risk, Injury, and Failure were defined as SCr above 150%, 200%, and 300%, respectively, of the median of age-specific SCr reference values. Continuous data are expressed as median (interquartile range (IQR)) and categorical data are expressed as number (%). *P*-values indicate overall comparison of all groups (that is, Non-AKI versus Risk versus Injury versus Failure). Intergroup differences were assessed using either [a]Kruskal-Wallis test or [b]Pearson χ^2 test, as appropriate. ^ Majority of the patients had severe PPHN. AKI, acute kidney injury; ICU, intensive care unit; PPHN, persistent pulmonary hypertension; SCr, serum creatinine.

	RIFLE classification				
	Non-AKI	**Risk**	**Injury**	**Failure**	
	n=89 (36%)	**n=72 (30%)**	**n=55 (23%)**	**n=26 (11%)**	
	Median (IQR)	*Median (IQR)*	*Median (IQR)*	*Median (IQR)*	***P-value***
Birth weight *(kilograms)*	3.3 (2.9-3.7)	3.4 (3.0-3.7)	3.3 (2.9-3.7)	3.0 (2.5-3.6)	0.242[a]
Apgar score					
1 minute after birth	*6 (3-8)*	*5 (3-7)*	*5 (3-7)*	*4 (1-7)*	*0.616*[a]
5 minutes after birth	*7 (5-9)*	*7 (5-8)*	*7 (5-8)*	*6 (5-8)*	*0.815*[a]
10 minutes after birth	*7 (6-8)*	*7 (6-8)*	*7 (4-8)*	*7 (6-8)*	*0.904*[a]
Age at start ECMO *(hours)*	44 (27-104)	36 (21-45)	38 (26-59)	38 (28-64)	***0.004***[a]
ECMO duration *(days)*	4.9 (3.6-8.2)	6.4 (4.2-10.2)	5.7 (4.4-8.9)	7.8 (4.5-15.1)	***0.012***[a]
Length of ICU stay *(days)*	8 (6-8.2)	11 (7-20.8)	10 (6-18)	14.5 (5.8-22.3)	0.178[a]
	n (%)	*n (%)*	*n (%)*	*n (%)*	***P-value***
Sex *Female*	41 (46%)	30 (42%)	25 (45%)	15 (58%)	0.576[b]
Diagnosis					0.155[b]
*Meconium aspiration syndrome^*	*40 (45%)*	*35 (49%)*	*22 (40%)*	*6 (23%)*	
*Congenital diaphragmatic hernia^*	*22 (25%)*	*22 (30%)*	*13 (24%)*	*13 (50%)*	
Isolated PPHN	*9 (10%)*	*8 (11%)*	*6 (11%)*	*3 (12%)*	
Other	*18 (20%)*	*7 (10%)*	*14 (265%)*	*4 (15%)*	
Before ECMO					
Vasopressor drugs	*81 (91%)*	*70 (97%)*	*50 (91%)*	*24 (92%)*	*0.535*[b]
Nitric oxide ventilation	*80 (90%)*	*53 (74%)*	*27 (49%)*	*14 (54%)*	***<0.001***[b]
Perinatal Asphyxia	*48 (54%)*	*40 (56%)*	*39 (71%)*	*14 (54%)*	*0.142*[b]
Cardiac arrest	*5 (6%)*	*5 (7%)*	*14 (26%)*	*7 (27%)*	***<0.001***[b]
During ECMO					
Major surgery	*4 (4%)*	*4 (6%)*	*8 (15%)*	*2 (8%)*	*0.137*[b]
Myocardial stunning	*3 (3%)*	*4 (6%)*	*0 (0%)*	*1 (4%)*	*0.385*[b]
Nonsurvivors	20 (22%)	13 (18%)	13 (24%)	17 (65%)	***<0.001***[b]
Early nonsurvivors <24h postECMO	*9 (45%)*	*4 (31%)*	*8 (62%)*	*9 (53%)*	
Late nonsurvivors ≥24h postECMO	*11 (55%)*	*9 (69%)*	*5 (38%)*	*8 (47%)*	

venovenous ECMO, but because of insufficient circulatory support, converted to veno-arterial ECMO. Eighteen (7%) patients underwent major surgery during ECMO, including surgical repair of diaphragmatic hernia or congenital heart defect.

Renal function and AKI

The median number of SCr measurements while receiving ECMO was 6 (IQR 4 – 9) per patient. Evidence of AKI while on ECMO had been documented for 153 (64%) patients, with 72 in the Risk category, 55 in the Injury category, and 26 in the Failure category (30%, 23% and 11% of all ECMO-treated patients, respectively). Of the 26 patients in the Failure group, 10 (38%) received continuous HF in addition to ECMO support for severe metabolic derangement and fluid excess unresponsive to diuretic therapy, as reflected by progressive oedema, ongoing oliguria, and hypertension. The median ECMO day at which patients reached the highest RIFLE category for the first time was day 2 (IQR 1 – 4) for Risk, day 2 (IQR 1 – 5) for Injury, and day 1 (IQR 1 – 3) for Failure. Of all 153 AKI patients, only 71 (46%) improved at least one RIFLE category, of whom 35 (49%), 30 (42%), and 6 (9%) initially classified as Risk, Injury and Failure, respectively. An overview of the AKI evolution is provided in Figure 2. The median duration from the highest RIFLE category

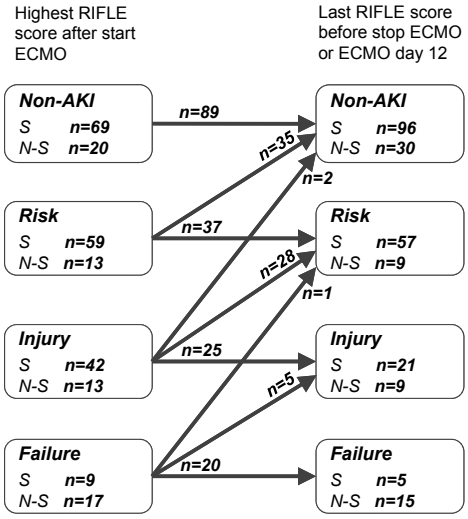

Figure 2. Evolution of acute kidney injury during ECMO.
Flow diagram showing the evolution of acute kidney injury (AKI) during treatment with ECMO. After the start of ECMO, all patients were stratified according to the highest RIFLE score attained. RIFLE categories Risk, Injury, and Failure were defined as, respectively, SCr above 150%, 200%, and 300%, of the median of age-specific SCr reference values. Subsequently the AKI evolution over time was evaluated by using the last RIFLE score before the cessation of ECMO or on ECMO day 12. All arrows indicate the direction of AKI evolution. Of all 153 AKI patients, only 71 (46%) patients improved at least one RIFLE category. NS, nonsurvivor; SCr, serum creatinine; S, survivor.

until the start of improvement was 2 days (IQR 1 – 5) for Risk, 2 days (IQR 1 – 3) for Injury, and 4 days (IQR 3 – 6) for patients with Failure. At the end of the study period only 126 patients (52%) out of the total pool of 242 patients were classified as Non-AKI.

In univariate analysis, patients with evidence of AKI were younger at the start of ECMO treatment (P=0.004). Patients who had suffered a cardiac arrest before ECMO were more prone to develop AKI (P<0.001), whereas no correlation was found with perinatal asphyxia or diagnosis category. The pre-ECMO use of NO ventilation was associated with a lower incidence of AKI (P<0.001). Major surgery or myocardial stun during ECMO, did not correlate with the occurrence of AKI. Thus, variables considered for the multivariate analysis included younger age at start ECMO, cardiac arrest, and NO ventilation pre-ECMO. By using multiple logistic regression, we found that only younger age at the start of ECMO treatment (P=0.004) and lack of NO ventilation (P=0.04) remained significant predictors of AKI. Patients with AKI had a longer ECMO duration (P=0.012) whereas no significant increase was noted regarding the length of ICU stay.

The survival rate in patients without AKI was 78% and decreased to 35% in the Failure category (Table 1 and Figure 3). Figure 4 shows the distribution of RIFLE categories on each ECMO day, according to survival. Comparing survivors with nonsurvivors regarding

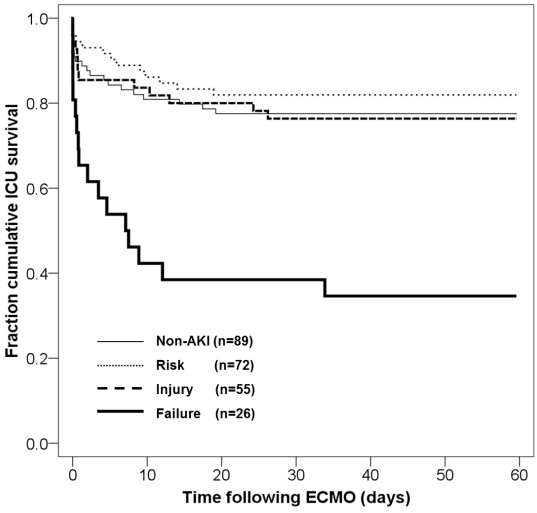

Figure 3. Kaplan-Meier survival curves stratified by RIFLE category.
All patients are stratified according to the highest RIFLE score attained during ECMO. Kaplan-Meier analysis estimates, for each RIFLE category, the rate of survival until intensive care unit (ICU) discharge among all patients after the cessation of ECMO. RIFLE categories Risk, Injury, and Failure were defined as, respectively, SCr above 150%, 200%, and 300%, of the median of age-specific SCr reference values. The differences between the Failure category and each of the other RIFLE categories are significant (all P<0.001; log-rank test). No significant differences were found between the Non-AKI, Risk and Injury categories. SCr, serum creatinine.

the distributions of RIFLE categories on the different ECMO days, we found that survivors generally had a better (lower) RIFLE score. *P*-values for day 1 to day 12 were significant (all $P<0.05$) except for days 9, 10 and 12 (Figure 4). RIFLE was predictive for both early (<24h after ECMO) and late non-survivors (>24h after ECMO) ($P<0.001$), but could not discriminate between these two outcomes ($P=0.43$). Besides RIFLE score, ECMO duration, and age at the start of ECMO were significantly related to mortality in univariate analysis. Longer ECMO durations and younger age were generally associated with higher mortality (both $P<0.005$). Diagnostic categories also were predictive for mortality ($P<0.001$). The mortality rates for patients diagnosed with PPHN, MAS, CDH, and the remaining group, "other" were 4%, 5%, 63%, and 30%, respectively. Simultaneous evaluation by using multiple logistic regression showed that RIFLE score ($P=0.003$), diagnostic category ($P<0.001$), and ECMO duration ($P<0.001$) were all independently related to mortality, whereas no significant effect of age remained. Survival until ICU-discharge was significantly lower among Failure patients. As a result, the adjusted odds ratio of mortality for Failure in comparison with the other RIFLE categories combined was 12.7 (95% CI, 3.3 to 49.5; $P<0.001$). No significant differences in survival were found between Non-AKI and the RIFLE categories Risk and Injury ($P=0.58$).

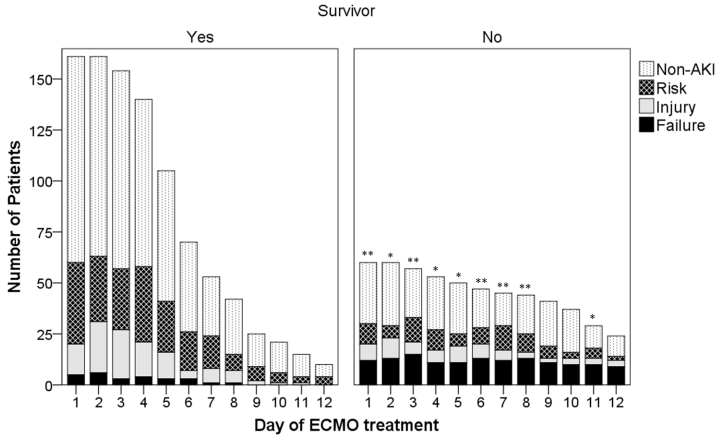

Figure 4. RIFLE distribution per day of ECMO treatment grouped according to survival.
The distribution of RIFLE categories on each ECMO day for patients who survived until intensive care unit (ICU) discharge compared with patients who did not survive until ICU discharge. RIFLE categories Risk, Injury, and Failure were defined as, respectively, SCr above 150%, 200%, and 300%, of the median of age-specific SCr reference values. Differences in RIFLE distribution per ECMO day between survivors and non-survivors were assessed by using Mann-Whitney U tests; **P*<0.05; ***P*<0.01. Survivors generally had a better RIFLE category throughout ECMO. SCr, serum creatinine.

DISCUSSION

To our knowledge, this is the first study in a large cohort of ECMO-treated neonates evaluating the incidence rate of AKI, systematically classified by the SCr-based RIFLE criteria. On each day of ECMO treatment, patients were assigned SCr-based RIFLE scores by using the actual SCr values in relation to the median of locally collected age-specific SCr reference values, as recently published by our institution (17). These reference values allowed us to reliably identify patients as having various degrees of AKI, despite the considerable changes in SCr due to the influence of maternal SCr and the rapidly changing renal function throughout the first weeks of life, even irrespective of ECMO treatment. Furthermore, to collect a homogeneous study group, we excluded patients who were prophylactically treated with HF during ECMO for fluid management as part of standard clinical care. Moreover, we excluded patients with congenital renal malformations.

The incidence of AKI was 63% for the RIFLE scores Risk, Injury and Failure; and 34% when exclusively evaluating the categories Injury and Failure. These results differ from those of other studies focusing on ECMO-treated patients (12, 14).

One study reported, in a very large cohort of approximately 10,000 ECMO patients, a much lower AKI incidence rate, varying from 8.0% in neonatal patients to 20.5% in pediatric patients (14). This discrepancy can be explained as follows. First, data were retrieved from the ELSO Registry database, which traditionally collects cross-sectional information, with the possibility that the AKI episode has been missed. Second, AKI was defined by using the renal complication code, as stated in the ELSO Registry (SCr >1.5 mg/dL). Although in newborns, this cut-off value may include lower grades of AKI, in older infants, this definition corresponds only to severe cases of the RIFLE category Failure. Finally, 27% of all patients received RRT according to hospital practice, of which further data on indication, timing, and dose are not provided. Standard HF may have prevented many patients from ever developing AKI, defined as SCr levels >1.5 mg/dL. A study by Smith and colleagues demonstrated a higher incidence of 71.7% of acute renal failure in 45 pediatric cardiac patients requiring ECMO. AKI severity was classified by an adapted pRIFLE score, with criteria for fluid retention added (20). In this study, case selection is certainly an important selection bias, as only the most complicated cardiac cases received ECMO. Moreover, the incidence rate of AKI may have been overestimated, as the category Failure also included those patients treated with HF to reduce fluid retention and electrolyte disturbances.

Another study, in 68 neonates with CDH requiring ECMO, reported an AKI incidence of 70.6% (12). The focus on solely CDH patients, with their compromised circulation, may explain this high incidence. In our CDH patients the AKI incidence was comparable at 69%.

We identified two clinical factors associated with AKI. The favorable effect of NO ventilation before ECMO might suggest a protective role against AKI. One explanation to think of is the systemic effect of inhaled NO, which includes modulation of the distribution of systemic blood flow and thereby potentially of renal perfusion. However, evidence on how changes in renal perfusion are related to the development of AKI is contradictory (21). Another predictor for AKI is younger age at the start of ECMO. Patients who were younger at the start of ECMO may have been the sicker ones, as they were not responding to conventional therapy earlier in life.

Overall, the severity of AKI is maximal within the first 2 days following the start of ECMO. As a consequence, we cannot exclude pre-ECMO renal injury, as the majority of patients were in need of vasopressor drugs. The high SCr levels throughout the first ECMO days contrast the expected fall in SCr due to dilution of the patients' blood by the extracorporeal volume. The clinical course of AKI is even more concerning, as only 46% of all patients initially classified as AKI show some degree of renal recovery during ECMO. In our study, survivors generally had a better RIFLE score than non-survivors. This is in agreement with a higher survival rate (78%) in patients without AKI compared with 35% in those with kidney failure. The adjusted odds ratio of mortality for Failure in comparison with the other RIFLE categories combined was 12.7 (95% CI, 3.3 to 49.5; $P<0.001$). This high mortality risk confirms the previously reported association between AKI and mortality (6, 11-14, 20, 22), and supports the idea that patients may benefit from early recognition of AKI and prevention of deterioration of renal function. With the high incidence of AKI in the present study, we should start worrying that many of these children could develop chronic kidney disease (CKD) in the long run (23-25).

Several limitations of this study should be addressed. First, SCr level is a delayed measure of decreased kidney function after AKI and is not very sensitive. Reference values vary widely during the first days of life, in particular with the risk of overestimation of AKI. Conversely, the ECMO circuit in neonates doubles the circulating volume, thereby diluting SCr levels. Hence, with SCr, the true incidence of AKI during the first days of ECMO treatment is hard to establish. A second limitation of our study is that we were not able to use urine output for grading AKI severity, which may have resulted in different incidences of Risk, Injury, and Failure.

The expected first sign of AKI associated with circulatory failure in ECMO candidates, tubular damage due to ischemia, may be detected early by the use of biomarkers in the urine, which are not affected by age or renal maturation, increased circulating volume or the ECMO circuit itself. These biomarkers may prove to be more sensitive, to enable the early diagnosis of AKI and may distinguish between potential AKI causes (26-29).

CONCLUSIONS

This study shows that the incidence of AKI in a large population of ECMO-treated neonates is remarkably high and that the severity of AKI is associated with mortality. Because AKI during childhood may predispose for CKD in adulthood, long-term follow-up of kidney function after ECMO is recommended.

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5 Loop diuretics are an independent risk factor for acute kidney injury in children on extracorporeal membrane oxygenation with pre-emptive continuous hemofiltration

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ABSTRACT

Purpose: Our aim was to systematically evaluate the prevalence and risk factors of acute kidney injury (AKI) following extracorporeal membrane oxygenation (ECMO) in patients who also received pre-emptive continuous hemofiltration (CH) as standard of care.

Methods: In this cohort study all children treated with neonatal (ECMO onset ≤28 days) or pediatric (ECMO onset >28days up to 18 years) ECMO and pre-emptive CVVH between 2007 and 2010 were included. AKI was defined using the RIFLE classification. SCr levels up to 3 days post-ECMO were compared to age-specific SCr reference values and patients were assigned to RIFLE categories (Risk, Injury, or Failure as 150%, 200% or 300% of median SCr reference value). Associations between AKI and clinical factors were explored with ordered logistic regression analysis.

Results: Eighty patients were included in the study (46 (58%) neonatal ECMO versus 34 (42%) pediatric ECMO). Twenty-nine of all patients (36%) met the criteria for AKI within three days following ECMO; 10 patients were classified as Risk, 8 patients as Injury and 11 patients as Failure. All but two of 60 survivors (97%) evaluated for renal recovery had normal renal function at discharge or transfer. In multivariate analysis only diuretic dose administered during ECMO was significantly associated with AKI post-ECMO (P=0.013).

Conclusions: The prevalence of AKI post-ECMO is remarkably high. Recovery of renal function, however, occurs in most survivors. The use of diuretics during ECMO should be closely monitored since AKI in the context of hypovolemia may be aggravated by excessive fluid removal by additional diuretics.

INTRODUCTION

Extracorporeal membrane oxygenation (ECMO) is an advanced cardiopulmonary bypass technique applied in acute reversible respiratory- or cardiovascular failure. It carries great risk of acute kidney injury (AKI), which we and others reported in up to two-thirds of children on ECMO without pre-emptive renal replacement therapy (RRT) (1-5). Moreover, AKI has been associated with poor outcomes including mortality and signs of chronic kidney disease on the longer term (1-7). As such, AKI during ECMO in children remains a major concern.

Throughout ECMO, fluid balance management is challenging since most patients are already fluid overloaded (FO) prior to ECMO cannulation (8). The development of AKI with oliguria may worsen FO. In a recent study in ECMO-treated patients, FO at continuous RRT initiation for AKI was associated with a longer ECMO duration, longer intensive care unit (ICU) length of stay (LOS), and higher mortality (9). Conversely, reduction of FO was associated with improved lung function and shorter time to decannulation (10, 11).

Recommendations to reduce FO vary by center from fluid restriction to aggressive diuretic therapy and early institution or even pre-emptive use of RRT (9, 12). In ECMO patients FO is the most common indication to initiate RRT (43%), followed by AKI (35%), and FO prevention (16%) (13). Several studies in neonates and children on ECMO demonstrated improvement in cumulative FO with RRT (5, 14, 15). Our group subsequently showed that pre-emptive continuous hemofiltration (CH) during ECMO resulted in a shorter time on ECMO, reduced number of ventilator days after ECMO, and a fewer number of blood transfusions (12). Pre-emptive CH was therefore included in our institutional ECMO protocol from 2006 onwards.

There is reason for concern, however. CH affects serum creatinine (SCr) and urine output and may therefore mask the recognition of AKI until after decannulation from the ECMO circuit. This limits our ability to tailor therapy (e.g., restrictive fluid balance and/or avoidance of nephrotoxic drugs) to improve outcomes (16). At 24 hours post-ECMO, up to 81% of adults have AKI (17). The generalizability of these data for pediatrics is limited due to differences in underlying disease and renal maturation. We are not aware of reports on renal outcomes of ECMO patients receiving pre-emptive CH. In this study we therefore systematically evaluated the prevalence and risk factors of AKI following decannulation of ECMO in pediatric patients who also received pre-emptive CH as standard of care.

MATERIALS AND METHODS

Setting

This cohort study was performed at the ICU of the Erasmus MC – Sophia Children's Hospital in Rotterdam, The Netherlands. This level III ICU is one of the two designated centers providing ECMO in The Netherlands. ECMO treatment in our ICU is available since 1992, and is most often performed in newborns and young children. The Ethics Committee of Erasmus University Medical Center waived the need for ethics approval and informed consent.

Patients

Patients eligible for inclusion were all children neonatal (ECMO onset ≤28 days following birth) or pediatric (ECMO onset >28days following birth up to 18 years of age) receiving ECMO support in combination with pre-emptive CH between January 2007 and January 2010. Patients were not eligible if (1) they suffered from preexistent kidney or urinary tract anomalies; or (2) could not be weaned from ECMO or deceased within 3 days after decannulation; or (3) if inadequate clinical data on renal function post-ECMO were available. If a patient had undergone more than one ECMO run, only data related to the first run were used.

Primary outcome - Acute kidney injury

The primary outcome was the prevalence of AKI within 72 hours post-ECMO. This follow-up period was chosen as a reasonable washout period of the CH effect. Furthermore, over a longer period post-ECMO renal function is more likely to be subject to disease course or medical treatment. AKI was defined according to the highest SCr-based RIFLE score obtained. The RIFLE classification defines three grades of increasing AKI severity, including R (Risk for kidney injury), I (Injury to the kidney) and F (Failure of kidney function) (18). RIFLE outcome categories L (Loss of renal function) and E (End-stage renal disease) were not applicable, as the study period was restricted to 72 hours post-ECMO. Since reliable SCr baseline concentrations pre-ICU admission were lacking in most patients, actual SCr levels compared to the 50^{th} percentile of age-corrected reference values were used to compute RIFLE scores (19, 20). RIFLE categories Risk, Injury, and Failure were defined as, respectively, SCr above 150%, 200%, and 300% of the median SCr reference values (19, 20). SCr levels were measured daily as part of standard clinical care. SCr was assessed by enzymatic assay (Creatinine Plus; Roche Diagnostics, Branchburg, NJ) on a Hitachi 912 analyser (21). In the patients who developed AKI, recovery of renal function was assessed at ICU discharge or transfer in terms of complete, partial or absent. Complete was defined as the absence of AKI by the RIFLE criteria; partial as a decrease in RIFLE grade but still AKI.

Data collection

Data were retrieved from the hospital's patient data management system which stores all prospectively collected physiological parameters, laboratory results and therapeutic modalities, together with medical chart review. Collected patient data included demographic characteristics, clinical course, ECMO support details and laboratory tests. More specifically, sex, gestational age, birth weight, and Apgar scores at 1 and 5 minutes after birth (only in newborns) as well as age, body weight, and primary diagnosis were collected. To assess the severity of illness and organ dysfunction, the Pediatric Risk of Mortality II (PRISM II) score and Pediatric Index of Mortality II (PIM II) score were collected (22, 23). Data were collected on ECMO type (e.g., venoarterial (VA) or venovenous (VV)), ECMO course (e.g., ECMO duration, pump flow), and administered drugs (e.g., antibiotics, diuretics) and drug plasma concentrations, if available. Vasopressor scores were calculated as follows: (dopamine dose (μg/kg/min) x 1) + (dobutamine dose (μg/kg/min) x 1) + (noradrenaline ((μg/kg/min) x 100) + (adrenaline ((μg/kg/min) x 100) (24). Furthermore, duration of mechanical ventilation, LOS, and survival until discharge were noted.

Standard of clinical care

ECMO support was started by the clinical team in patients suffering from potentially reversible cardio-respiratory diseases unresponsive to conventional intensive care; with an expected mortality rate greater than 80% using the criteria reported by Stolar (25, 26). The ECMO circuit was primed and pre-emptive CH had been installed according to a hospital-based protocol as described before (12). Ultrafiltration was targeted to maintain a daily neutral or negative fluid balance depending upon the patients' clinical condition. Diuretics were prescribed at the attending physician's discretion if a targeted fluid balance or urine output of >0.5 ml/kg/hour could not be achieved. During the study period, institutional policy regarding ECMO support did not change. Therapeutic drug monitoring for antibiotics was routinely performed according to clinical guidelines.

Data analysis

All data are expressed as median values with interquartile range (IQR) for continuous variables or numbers with percentages for categorical variables, unless indicated otherwise. 95% Binomial confidence intervals (CI) were calculated with the Agresti-Coull method. Univariate comparisons were performed using Kruskal-Wallis test, Pearson's chi-square or Fisher's exact test to explore associations between the primary renal outcome groups (that is, non-AKI versus Risk versus Injury versus Failure) and clinical factors related to ECMO support; i.e. age at start of ECMO, PRISM II score, ECMO mode, ECMO duration, and diuretic dose administered. Diuretics prescribed were either furosemide or bumetanide. To convert a bumetanide dosage to a furosemide dosage, the

bumetanide dose was multiplied by 40, to correct for the difference in potency (27). The sum of both was used to indicate the furosemide equivalent dose administered (mg/kg/ECMO day). Significant univariate associations were then entered into a multivariable ordinal logistic regression model to identify independent associations. In this regression model, the dependent variable was the ordered variable renal status, with categories non-AKI, Risk, Injury, and Failure. Survival post-ECMO until ICU-discharge for non-AKI versus AKI was evaluated using a log-rank test. Two-sided P=0.05 was considered the limit of significance in all analyses. Data were analysed using IBM SPSS statistics version 20 (Statistical Package for the Social Sciences, Chicago, IL).

RESULTS

Patient characteristics

From 2007 to 2010, 105 children were treated with ECMO and pre-emptive CH. Twenty-five of them were excluded: for five patients data on renal outcome were inadequate; five had preexistent renal anomalies diagnosed on ultrasound; 15 patients could not be weaned from ECMO and died at a median of 4 hours (IQR 2 – 5) after decannulation. As a result, 80 patients were included (46 (58%) neonatal ECMO support versus 34 (42%) pediatric ECMO) as shown in Figure 1. Table 1 shows detailed patient characteristics

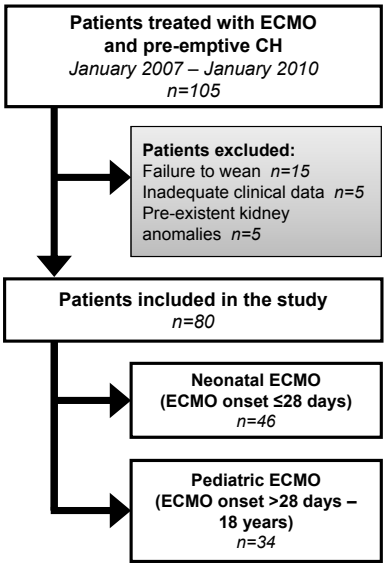

Figure 1. Flowchart of patient recruitment.
ECMO, extracorporeal membrane oxygenation; CH, continuous hemofiltration.

Table 1. Baseline characteristics.
Patients were enrolled according to type of ECMO support which is neonatal ECMO (ECMO onset ≤28 days) versus paediatric ECMO (ECMO onset >28days up to 18 years of age). Continuous data are expressed as median (interquartile range (IQR)) and categorical data are expressed as number (%).
ICU, intensive care unit; PIM, Paediatric Index of Mortality; PRISM II, Paediatric Risk of Mortality Score III; RRT, renal replacement therapy; VA, venoarterial; VV, venovenous.

	All patients (n=80)		**Neonatal ECMO (n=46)**		**Pediatric ECMO (n=34)**	
Gestational age, *weeks*	39.9	(36.9 – 40.1)	39.6	(37.0 – 40.6)	40.0	(34.0 – 40.0)
Birth weight**,** *kilograms*	3.3	(2.4 – 3.5)	3.2	(2.6 – 3.7)	3.5	(2.0 – 3.5)
Age at start ECMO, *days for all patients*, *hours for neonatal ECMO, years for pediatric ECMO*	9.2	(1.8 – 211)	53	(33 – 130)	1.80	(0.30 – 6.1)
Weight at start ECMO	3.9	(3.0 – 6.9)	3.0	(2.7 – 3.8)	10.5	(4.3 – 25.3)
Gender, *male*	42	(53)	22	(48)	20	(59)
Diagnosis						
Congenital diaphragmatic hernia	*16*	*(20)*	*16*	*(35)*	-	
Meconium aspiration syndrome	*13*	*(16)*	*13*	*(28)*	-	
Sepsis	*12*	*(15)*	*7*	*(15)*	*5*	*(15)*
Cardiac disease	*13*	*(16)*	*5*	*(11)*	*8*	*(24)*
Pneumonia	*14*	*(18)*	-		*14*	*(41)*
Other	*12*	*(15)*	*5*	*(11)*	*7*	*(20)*
Severity of illness at ICU admission						
PIM II, %	*11*	*(4 – 25)*	*10*	*(4 – 24)*	*11*	*(5 – 25)*
PRISM II, %	*18*	*(13 – 23)*	*19*	*(9 – 36)*	*16*	*(11 – 24)*
Time on mechanical ventilation, *days*	15	(10 – 35)	16	(9 – 36)	15	(10 – 32)
ECMO duration**,** *hours*	129	(78 – 192)	123	(83 – 183)	159	(64 – 196)
Length of ICU stay**,** *days*	21	(10 – 40)	23	(11 – 48)	20	(10 – 37)
ECMO course						
ECMO mode						
Venoarterial (VA)	*52*	*(65)*	*34*	*(74)*	*17*	*(50)*
Venovenous (VV)	*25*	*(31)*	*12*	*(26)*	*14*	*(31)*
Convversion - VV to VA	*3*	*(4)*	-		*3*	*(9)*
Vasopressor score						
Before start ECMO	*69*	*(30 – 92)*	*80*	*(45 – 101)*	*40*	*(10 – 87)*
After start ECMO	*17*	*(6 – 38)*	*20*	*(9 – 38)*	*10*	*(5 – 42)*
Pumpflow, *ml/kg/min*						
4h postcannulation	*100*	*(75 – 128)*	*111*	*(96 – 135)*	*86*	*(58 – 113)*
24h postcannulation	*98*	*(71 – 129)*	*113*	*(87 – 134)*	*82*	*(51 – 121)*
Aminoglycoside antibiotics						
Yes	78	(98)	45	(98)	33	(98)
Loop diuretics						
Furosemide	*48*	*(60)*	*27*	*(59)*	*21*	*(62)*
Bumetanide	*2*	*(3)*	-		*2*	*(6)*
Furosemide / Bumetanide combination	*15*	*(19)*	*11*	*(24)*	*4*	*(12)*
Survival until ICU discharge	62	(78)	32	(70)	30	(88)

stratified by age (neonatal versus pediatric ECMO). Of all enrolled patients, 42 (51%) were male. The median age and body weight at the start of neonatal ECMO were 53 hours (IQR 33 – 130) and 3.0 kilograms (IQR 2.7 – 3.8), respectively, versus 1.8 years (IQR 0.3 – 6.1) and 10.5 kilograms (IQR 4.3 – 25.3) at start of pediatric ECMO. In neonates the most common primary diagnoses were congenital diaphragmatic hernia (CDH) (n=16; 35%) and meconium aspiration syndrome (MAS) (n=13; 28%). The most common primary diagnoses in older children were pneumonia (n=14; 41%) and cardiac disease (n=8; 24%).

Of all patients, 51 (64%) received VA ECMO. Three of the 29 (10%) patients initially placed on VV ECMO were converted to VA ECMO due to ongoing hemodynamic instability unresponsive to fluid resuscitation and medication. Patients were weaned from ECMO after a median of 129 hours (IQR 78 – 191). Finally, 18 (23%) patients died at a median of 18 days (IQR 5 – 26) after ECMO. Most of the nonsurvivors were neonatal patients diagnosed with CDH (n=9, 50% of the nonsurvivors).

Nephrotoxic antibiotics including gentamycin and vancomycin were administered for suspected or proven infection in 78 (98%) patients. Sixty-five (81%) patients received loop diuretics during ECMO, 48 (60%) furosemide only, two (3%) bumetanide only, and 15 (19%) patients received both. Moreover, 15 of these 65 (23%) patients received diuretics only once. The median furosemide equivalent dose in all 65 patients was 1.3 (0.6 – 3.7) mg/kg/ECMO day.

Renal outcome

Twenty-nine patients (36%, 95% CI, 27 – 47) met the criteria for AKI within three days after ECMO (Figure 2). Ten (34%) were classified as Risk, 8 (28%) as Injury and 11 (38%) as Failure. Of the latter, only one required prolonged RRT and was therefore placed on peritoneal dialysis for 8 days following ECMO. Renal function in this patient recovered and peritoneal dialysis was discontinued before hospital discharge. Survival rates did not significantly differ between non-AKI and AKI patients (66% vs. 84%, log-rank test, $P>0.05$).

Renal recovery before ICU discharge was evaluated in all survivors who initially developed AKI within 72 hours post-ECMO (n=19). In two patients initially classified as Injury, data on renal recovery were lacking due to early transfer on post-ECMO day 3 to referral hospitals. Kidney function completely and partially recovered in, respectively, 15 and 2 patients diagnosed with AKI who survived to ICU discharge (median LOS post-ECMO was 27 days (IQR 11 – 98). One Failure patient improved to Injury and one to Risk after an ICU stay of 26 and 23 days, respectively. Altogether, at ICU discharge 97% of all survivors (58/60) had SCr levels <150% of the median reference values, irrespective of their AKI status within 3 days post-ECMO.

Vancomycin and gentamycin serum trough concentrations were below toxic levels, except for one level of 22.8 mg/l in one patient on the first day after ECMO decannula-

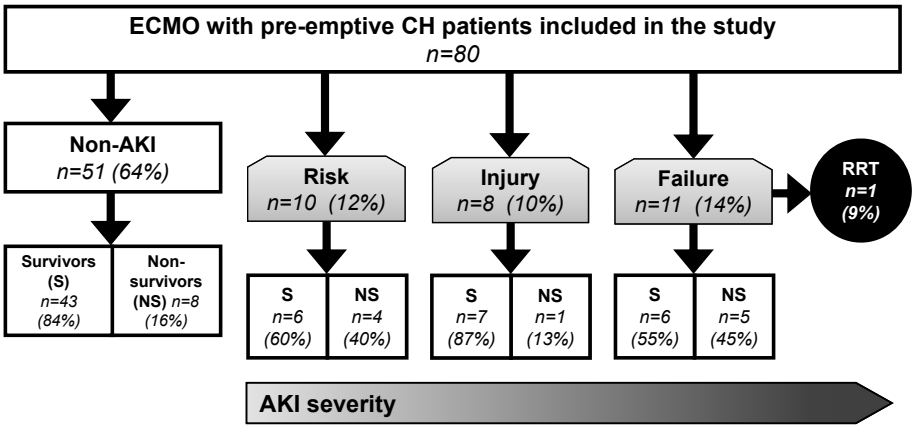

Figure 2. Maximal SCr based RIFLE score obtained following ECMO decannulation.
RIFLE categories Risk, Injury, and Failure were defined as, respectively, SCr above 150%, 200%, and 300%, of the median of age-specific SCr reference values. AKI, acute kidney injury; CH, continuous hemofiltration; NS, nonsurvivors; RRT, renal replacement therapy; SCr, serum creatinine; S, survivors.

tion (28). The vancomycin dosage was lowered according to the pharmacist-enforced protocol and SCr levels stayed within the normal limits at all time points.

ECMO mode (*P*=0.029) and furosemide equivalent dose administered (*P*=0.003) were associated with AKI post-ECMO in univariate analysis, whereas age at the start of ECMO (*P*=0.185), ECMO duration (*P*=0.111) and PRISM II (*P*=0.992) were not (Table 2). A multivariable logistic regression analysis including the two significant co-variates, revealed that only furosemide equivalent dose during ECMO was significantly associated with

Table 2. Variables associated with AKI post-ECMO.
This table represents the variables entered in univariate analysis. Continuous data are expressed as median (interquartile range (IQR)) and categorical data are expressed as number (%). *P*-values indicate overall comparison of all groups (that is, non-AKI versus AKI). Intergroup differences were assessed using either [a]Kruskal-Wallis test or [b]Pearson's Chi-Square test, as appropriate. AKI, acute kidney injury; ICU, intensive care unit; PRISM II, Paediatric Risk of Mortality Score III; RRT, renal replacement therapy.

	Non-AKI	**AKI**	
Variable	*Median, (IQR)*	*Median, (IQR)*	***P-value***
Age at start ECMO, *days*	9.2 (232 – 625)	9.4 (1.4 – 170)	0.516[a]
ECMO duration, *hours*	114 (79 – 178)	150 (74 – 237)	0.213[a]
Furosemide equivalent dose during ECMO, *mg/kg/ECMO day*	0.99 (0.41 – 1.95)	2.78 (0.87 – 6.71)	***0.003***[a]
Severity of illness at ICU admission, *PRISM II*	19 (12 – 23)	18 (14 – 23)	0.151 [a]
	n (%)	*n (%)*	***P-value***
ECMO mode			***0.041***[b]
Venoarterial	*30 (59)*	*24 (83)*	
Venovenous	*21 (41)*	*5 (17)*	

the AKI occurrence and severity (*P*=0.013, ECMO mode *P*>0.05). The odds ratio to AKI after ECMO was 1.3 (95% CI, 1.1 – 1.6, *P*=0.009) for patients receiving diuretics. Median furosemide equivalent dose was 1.0 (IQR 0.4 – 3.0) in patients without AKI and 1.2 (IQR 0.8 – 4.2), 1.8 (IQR 0.5 – 6.3), and 3.9 (0.7 – 6.8) mg/kg/ECMO day, respectively, in patients classified as Risk, Injury and Failure (Figure 3).

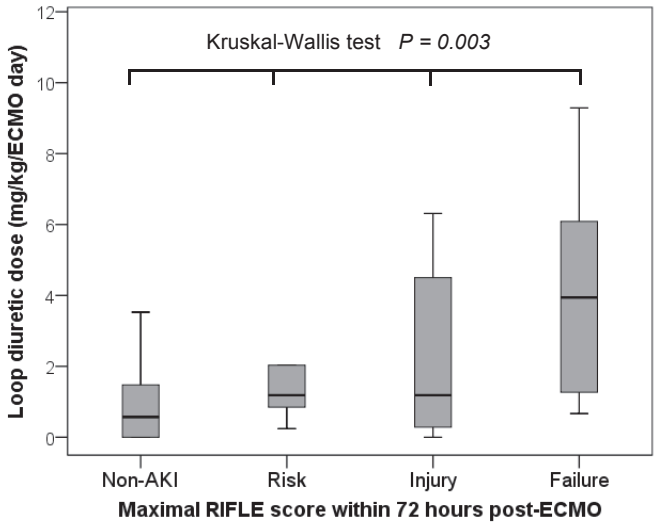

Figure 3. Furosemide equivalent dose administered during ECMO.
Diuretic dose administered during ECMO in milligram per kilogram per ECMO day (furosemide + (bumetanide *40)), according to the maximal SCr based RIFLE score obtained. RIFLE categories Risk, Injury, and Failure were defined as, respectively, SCr above 150%, 200%, and 300%, of the median of age-specific SCr reference values. The amount of diuretic drugs during ECMO was associated with AKI in the immediate post-ECMO period (Kruskal-Wallis test, *P*=0.003). AKI, acute kidney injury; SCr, serum creatinine.

DISCUSSION

To the best of our knowledge, this study presents the first systematic evaluation of AKI directly following neonatal and pediatric ECMO support with pre-emptive CH. Almost 40% of the patients developed AKI post-ECMO, classified as Risk in 10 (34%), Injury in 8 (28%) and Failure in 11 (38%) patients. We aimed to establish a homogeneous study cohort by excluding patients suffering from preexistent renal diseases. Moreover, throughout the study period, fluid management by CH, standardized for timing, modality, and dose, was similar in all ECMO patients. At ICU discharge or transfer, 97% of all ECMO survivors had normal renal function.

One study in pediatric cardiac patients receiving ECMO with or without early CH, compared post-ECMO SCr levels (29). They reported fairly high median post-ECMO SCr

levels of 62 µmol/L for patients with a median age of 13 to 57 days. Although actual AKI incidence or severity was not reported, this suggests that a large proportion of their patients suffered from AKI (29). Interestingly, a considerably higher AKI incidence of 81% was reported in adults admitted to a specialized cardiovascular surgery ICU, most likely due to the differences in underlying pathologic conditions between these adults versus children (17).

A few studies have been published on renal recovery following pediatric ECMO and 'therapeutic' CH. Short-term persisting renal insufficiency only seems to occur in children with preexistent renal disease (30, 31). The findings of the current study are consistent with these studies, despite the different indications to initiate CH. On the intermediate term, one study based upon chart review from inpatient and outpatient visits reported that none of 15 survivors of ECMO with CH had renal insufficiency (29). Longer-term data on renal function remain scarce.

Of all 80 patients enrolled in our study, only one patient required ongoing RRT following ECMO and was placed on peritoneal dialysis. This prevalence is low compared to the 42% and 26% reported in adult and pediatric patients after ECMO and CH, respectively, requiring RRT at 24 hours post ECMO (17, 31). This discrepancy may be due to different patient selections as previous studies included patients in whom CH during ECMO was initiated based on clinical reasons rather than pre-emptively. It would be interesting to learn how many of their patients who required ongoing RRT, initially met criteria for FO only.

In multivariable analysis furosemide equivalent dose administered during ECMO was the only covariate associated with AKI. Surprisingly, considering that CH was in place for fluid management, 65 patients (81% of all patients) received diuretics during ECMO of whom 50 (63% of all patients) more than once. In this context our data raise important questions about the clinical benefit of administering loop diuretics to patients receiving ECMO and pre-emptive CH. In critically ill non-ECMO adults, the use of diuretics during AKI was associated with higher risk of death and non-recovery of renal function (32). In children undergoing cardiac surgery with cardiopulmonary bypass, which is to some extent similar to ECMO, those with poor renal outcomes were 4-12 times more likely to have received diuretics (33).

We consider the following explanations for the association between diuretic dose and post-ECMO AKI. First, diuretics were prescribed for oliguria, which may have been due to unrecognized AKI or due to intravascular volume depletion as a result of fluid removal by CH. Excessive fluid removal, either by diuretics, CVVH or combination therapy, may increase the risk of pre-renal AKI, potentially followed by acute tubular necrosis (8). To unravel the complex relationship between fluid balance and renal function, one should prospectively evaluate fluid management including fluid restriction, diuretic regimens, and CH during ECMO.

Secondly, although loop diuretics are not considered a direct nephrotoxin, the addition of vancomycin may have increased the risk of developing nephrotoxicity (34). In our study 98% of our patients received nephrotoxic antibiotics. While elimination of loop diuretics is reduced in neonates as compared to adults, only one study in neonates treated with bumetanide and ECMO showed a greater steady-state volume of distribution and elimination half-life (35). This reflects the under documentation of diuretic pharmacokinetics, dosing regimens and associated outcomes in ECMO-treated children (36).

Several limitations of this study should be addressed. First, this was a single-center analysis with a relative small sample size. Second, AKI was diagnosed on the basis of SCr, which has inherent limitations to accurately estimate GFR (37). Moreover, we did not include urine output in the RIFLE criteria whereas this has been demonstrated beneficial to stage AKI in critically ill patients (38). Reason not to use urinary output data was, that urine volume on the day of ECMO decannulation is often low due to decreased cardiac output following decannulation (39). Finally, we did not evaluate the use of diuretics in a control group of children treated with ECMO without CH.

CONCLUSIONS

Our study is, to the best of our knowledge, the first to show that the prevalence of AKI immediately after ECMO with pre-emptive CH is remarkably high. Recovery of renal function, however, occurred in most survivors. Although this study cannot draw conclusions about cause-and-effect relationships, special attention must be paid to the administration of diuretics during ECMO with pre-emptive CH observing the independent association with AKI post-ECMO. Physicians should be aware that AKI in the context of hypovolemia may be precipitated by excessive fluid removal by additional diuretics.

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6 Urinary neutrophil gelatinase-associated lipocalin identifies critically ill young children with acute kidney injury following intensive care admission: a prospective cohort study

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ABSTRACT

Introduction: Children admitted to a pediatric intensive care unit (ICU) are at high risk of developing acute kidney injury (AKI). Although used in clinical practice, serum creatinine (SCr) is insensitive for early diagnosing AKI. Urinary neutrophil gelatinase-associated lipocalin (uNGAL) and kidney injury molecule-1 (KIM-1) are novel AKI biomarkers of which the performance in pediatric ICU patients is largely unknown. We aimed to characterize uNGAL and KIM-1 patterns in children following ICU admission and to assess their properties to identify children at risk for AKI development.

Method: From June 2010 until January 2014 we conducted a prospective observational cohort study of term-born children aged one day to one year on mechanical ventilation. Blood and urine samples were obtained every 6 to 12 hours up to 72 hours post-admission. Blood samples were assayed for SCr; urine samples for uNGAL and KIM-1. The RIFLE classification (Risk, Injury, or Failure as 150%, 200% or 300% of median SCr reference values) was used to define AKI.

Results: 100 children were included (80 survived). Median age at admission was 27.7 days (IQR 1.5–85.5); median duration of mechanical ventilation was 5.8 days (IQR 3.1–11.4). Thirty-five patients had evidence of AKI within the first 48 hours post-admission of whom 24 (69%) already had AKI when entering the ICU. uNGAL and KIM-1 concentrations in AKI peaked between 6 to 12 hours and 12 to 24 hours post-admission, respectively. The maximal ROC-AUC for uNGAL was 0.815 (95%CI 0.685-0.945, *P*-value <0.001) at 0 to 6 hours post-admission. The discriminative ability of KIM-1 was moderate with a largest AUC of 0.737 (95%CI 0.628-0.847, *P*-value <0.001) at 12 to 24 hours post-admission. At the optimal cut-off point (126 ng/mL), uNGAL concentration predicted AKI development correctly in 16 out of 19 (84%) children, up to 24 hours before a rise in SCr became apparent.

Conclusion: Levels of uNGAL and KIM-1 increase in patients with AKI following ICU admission and peak at 6 to 12 hours and 12 to 24 hours, respectively. uNGAL seems a reliable marker for identifying children who will develop AKI 24 hours later.

INTRODUCTION

Acute kidney injury (AKI) is a frequent and serious complication in critically ill children (1-7). Moreover, it has been shown an independent risk factor for mortality, prolonged length of intensive care unit (ICU) stay, and prolonged mechanical ventilation (1, 2, 5). Current consensus criteria for diagnosing AKI are based on changes in serum creatinine (SCr) and urine output (4, 8). One must realize however, that SCr is an indicator of glomerular function rather than a marker of renal tubular cell damage, which typically occurs during the initial phase of AKI in ICU patients (9, 10). In addition, a substantial number of functioning nephrons have to be compromised before changes in SCr levels become evident (11). Moreover, SCr is influenced by factors unrelated to renal function and in the newborn reflects maternal levels immediately after birth (11, 12). Altogether, SCr is increasingly considered a late and not very sensitive marker for diagnosing AKI.

Therefore, research has increasingly focused on the identification of novel more sensitive biomarkers for renal, especially tubular, injury, including urinary neutrophil gelatinase associated lipocalin (uNGAL) and kidney injury molecule-1 (KIM-1) (13). NGAL is a small 25 kDa protein initially discovered in activated human neutrophils (14). NGAL is expressed in limited quantities in other human tissues including the lungs, spleen and kidneys, where it is thought to inhibit bacterial growth, scavenge iron and induce epithelial growth (14-18). Plasma NGAL is freely filtered by the glomerulus and then largely reabsorbed by proximal tubular cells (19). Upon renal tubular injury, NGAL reabsorption may be decreased whereas NGAL de novo synthesis in epithelial cells of Henle's loop and of distal tubule segments is strongly upregulated after which it is found in high concentrations in the urine (20). KIM-1 is a 104 kDa type 1 transmembrane glycoprotein that contains both an immunoglobulin-like and a mucin domain in its extracellular portion (21). It is expressed in low levels in healthy proximal tubule cells and thought to promote apoptotic and necrotic cell clearance (22). Upon kidney ischemia or toxicity, KIM-1 is highly upregulated and shed into the extracellular space and urine (21, 22).

The usefulness of uNGAL was first recognized by Mishra and colleagues who demonstrated in children that postoperative uNGAL levels at 2 hours after cardiopulmonary bypass (CPB) had a nearly 100% accuracy for predicting AKI at 24 – 72 hours (23). Subsequent studies, mainly in adults, in clinical settings such as critical care and kidney transplantation, confirmed this finding (24, 25). KIM-1, on the other hand, has only been systematically investigated in patients undergoing CPB and in a small sample of asphyxiated neonates (26, 27). Zappitelli and colleagues were the first to evaluate uNGAL in a large heterogeneous group of PICU patients. It was found a good diagnostic marker for development of AKI and persistent AKI for ≥48 hours, but not for AKI if uNGAL had been measured after a rise in SCr (28). Later studies focused on cut-off points for AKI

prediction specifically in pediatric ICU patients, evaluated biomarker combinations, and even suggested uNGAL as a predictor for mortality (29, 30).

None of these previous studies, however, provided insight in the biomarker evolution using time-intervals based on hours shortly after ICU admission. Besides, these studies report data for subjects with widely varying age-ranges, from one week to 21 years (27-31). None, however, focused on children up to one year of age whereas this age group is particularly vulnerable to renal injury during the physiological evolution of renal function. Therefore, our aim was to characterize temporary uNGAL and KIM-1 patterns in the three days following ICU admission in a large cohort of critically ill children up to one year of age. Secondly, we aimed to assess whether levels of these biomarkers during the first 24 hours of admission can reliably identify children at risk for AKI development within 48 hours following admission.

METHODS

Setting

From June 2010 until January 2014 a single-center prospective observational cohort study was conducted in the level III ICU of the Erasmus Medical Center – Sophia Children's Hospital, Rotterdam, The Netherlands. Considered for enrolment were children (born >37 weeks of gestational age) between the ages of one day and one year admitted to the ICU and requiring endotracheal intubation and mechanical ventilation. Patients were not eligible for inclusion if (1) they had congenital abnormalities of the kidney or urinary tract, (2) death was anticipated within 24 hours, or (3) they received mechanical ventilation for other reasons (e.g. neuromuscular disease). Patients were excluded when treatment with extracorporeal membrane oxygenation was required within the study period. The study protocol was approved by the local medical ethical review board of the Erasmus Medical Center. The deferred consent process was used whereby written informed consent was obtained from the primary caregivers within 12 hours following the study start. The collected blood and urine specimens of those children for whom consent was withdrawn were destroyed (n=10 children, maximal 1.4 mL blood per patient).

Sample collection and analytical procedures

Upon ICU admission blood and urine samples were prospectively collected concomitantly between 0 – 6 hours (T0), 6 – 12 hours (T1), 12 – 24 hours (T2), 24 – 36 hours (T3), 36- 48 hours (T4), and 48 – 72 hours (T5) of admission. Per time-frame 0.7 mL blood was drawn from an indwelling arterial line, if available, or by capillary or venous puncture. Urine samples were collected using a bladder catheter. To collect 3 mL freshly voided

urine, the urine collection bag was emptied one hour prior to each sampling time frame. Urine samples were left refrigerated for sedimentation for 2 to 3 hours, aliquoted and stored within four hours after collection at -80ºC until the assay.

Creatinine concentrations were measured in the hospital's clinical chemical laboratory by an enzymatic assay (Creatinine Plus; Roche Diagnostics, Banchburg, NJ) on a Cobas 8000 analyzer. During the period of sample collection, the interassay coefficient of variation (CV) was less than 2.6%.

uNGAL was measured using the latest uNGAL chemiluminescent microparticle immunoassay developed for a standardized clinical platform (ARCHITECT analyzer, Abbott Diagnostics Division, Abbott Laboratories, Abbott Park, IL, USA). The mean inter-assay coefficient of variation for uNGAL was 5.3% at a concentration of 19.4 ng/mL. The limit of quantification (LoQ) of uNGAL was 3.0 ng/mL while the upper limit of quantitation was 6000 ng/mL. The reagents and calibrator for the uNGAL assays were kindly supplied by Abbott Diagnostics (Abbott Laboratories, Abbott Park, IL, USA). KIM-1 was measured using a commercially available enzyme-linked immunosorbent assay kit (ELISA) (BioAssay Works, Ijamsville, MD, USA). The mean inter-assay coefficients of variation for KIM-1 was <14% at a concentration of 0.17 ng/mL. The LoQ of KIM-1 was 0.08 ng/mL. Levels of uNGAL and KIM-1 were expressed in absolute values (ng/mL).

Data collection

Recorded patient data included clinical characteristics and results of laboratory tests. More specifically, gender, gestational age, birth weight as well as age, body weight, and diagnosis at admission were collected. The Pediatric Risk of Mortality II (PRISM II) score and Pediatric Index of Mortality II (PIM II) score were collected as an indication of severity of illness (32, 33). Furthermore, cardiac arrest on ICU admission and type of mechanical ventilation were registered together with the fraction of inspired oxygen at the time of intubation, the need for Nitric Oxide ventilation, and the administration of vasopressor drugs, diuretics (furosemide or bumetanide) or aminoglycosides (gentamicin, tobramycin or amikacine). Lastly, data on the following outcomes were collected: treatment with renal replacement therapy, duration of mechanical ventilation, lengths of ICU and hospital stay, and survival until ICU discharge.

Definitions

AKI was defined according to the maximal SCr-based RIFLE score obtained within the first 48 hours following admission. The RIFLE classification defines three grades of increasing AKI severity, including R (Risk for kidney injury), I (Injury to the kidney), and F (Failure of kidney function) (8). RIFLE outcome categories L (Loss of renal function) and E (End-stage renal disease) were not applicable, as the study was restricted to the first seven days following ICU admission. Since all children enrolled were younger than

one year of age, most did not have baseline SCr concentrations pre-ICU admission nor is there an algorithm available to calculate eGFR, which is why RIFLE categories Risk, Injury, and Failure were defined as SCr concentrations above 150%, 200%, and 300%, respectively, of the median age-specific SCr reference value. These SCr reference values were obtained from a large cohort of children without kidney disease, by using small age intervals ranging from 1 day in the first week after birth up to 3 months at the end of the first year of age (34). 'Persistent AKI' was defined as lack of improvement of RIFLE score within 72 hours post-admission.

Statistical analysis

Unless indicated otherwise, continuous data are expressed as median values with inter-quartile range (IQR) and discrete data as numbers with percentages (%). Patients were grouped according to whether they lacked AKI or had AKI (either RIFLE score Risk, Injury or Failure) within 48 hours following admission. Clinical characteristics and biomarker levels were compared between AKI- and non-AKI patients using univariate analyses for continuous variables (Mann-Whitney U test) and categorical variables (Pearson's chi-square test or Fisher's exact test, as appropriate). Biomarker levels were also compared between RIFLE strata and diagnosis categories using univariate overall comparisons between groups (Kruskal-Wallis tests). Receiver operating characteristics (ROC) curves were generated for the occurrence of AKI within 48 hours following intubation using biomarker levels at three different time frames (T0, T1, and T2, respectively, 0 – 6 hours, 6 – 12 hours, and 12 – 24 hours) as well as 24 hours peak levels. The areas under the curve (AUC), with 95% confidence intervals (95% CI), were calculated. Also, for each time frame the optimal cut-off value based on the Youden index was calculated with corresponding sensitivity and specificity. Using those cut-off values, sensitivity and specificity of both biomarkers for predicting AKI, as well as the positive and negative predictive values, were calculated for patients who developed AKI later on within the study period (<72 hours post-admission) after being considered AKI-free on admission. Of these patients, the timing and absolute values of maximum biomarker levels in urine samples preceding AKI were compared to biomarker levels in the first urine samples of controls (non-AKI critically ill children). Two-sided P=0.05 was considered the limit of significance in all analyses. Data were analyzed using IBM SPSS statistics version 21 (Statistical Package for the Social Sciences, Chicago, IL).

RESULTS

Patients

One-hundred and ten patients were initially included by deferred consent. However, since consent was withdrawn by ten (9%) parents, 100 patients were enrolled in the study. Table 1 details the characteristics for all patients as well as stratified by occurrence of AKI within 48 hours post-admission. Median age and body weight at ICU admission were 27.7 days (IQR 1.5 – 85.5) and 3.8 kilograms (IQR 3.2 – 5.3), respectively. The most common primary diagnoses were congenital diaphragmatic hernia (n=23, 23%) and respiratory failure (n=20, 20%). For all patients the median duration of mechanical ventilation was 5.8 days (IQR 3.1 – 11.4) and the median ICU stay was 10.0 days (IQR 6.1 – 27.0). Seventeen patients (17%) died during admission after a median ICU stay of 11.7 days (IQR 4.4 – 27.2).

Acute kidney injury

Thirty-five patients (35%) met the criteria for AKI within 48 hours following ICU admission. Fifteen of those (42%) were classified as RIFLE-R, 10 (29%) as RIFLE-I and 10 (29%) as RIFLE-F. One patient classified as RIFLE-F received renal replacement therapy, starting two days post-admission. Twenty-four of the 35 AKI patients (69%) already met AKI criteria at the time of admission (Risk n=6, 25%; Injury n=8, 33%; Failure n=10, 42%).

Age, weight, diagnosis, and cardiac arrest experienced on ICU admission did not significantly differ between patients who developed AKI within 48 hours following admission and those who did not (Table 1). Nonetheless, the former were more severely ill on admission as reflected by a significantly higher PRISM II score (48.9 (IQR 25.5 – 77.1)) than that assigned to non-AKI patients (25.1 (IQR 10.10 – 54.1); *P*-value <0.001 by Mann-Whitney U test). Moreover, AKI patients were ventilated almost twice as long although type of mechanical ventilation, fraction of inspired oxygen, and the need for Nitric Oxide ventilation did not differ. AKI patients received more often two or more types of vasopressor drugs at intubation and had a longer ICU- and hospital length of stay. In contrast, there was no difference in the prescription of diuretic drugs and aminoglycosides between AKI and non-AKI patients. Lastly, the overall mortality rate in AKI patients was significantly higher than that of non-AKI patients (32% versus 14%; *P*-value=0.027 by Pearson's chi-square test).

Biomarker patterns post-admission

In total 491 urine samples were collected, i.e. 86% of all scheduled samples (median 5 (IQR 4 – 5) samples per patient). Sampling was not feasible in cases of anuria, discontinuation of bladder catheterization, or logistical problems. Of all urine specimens, the median uNGAL concentration was 39.5 ng/mL (IQR 12.4 – 168.1 ng/mL) and the median KIM-1 concentration was 0.14 ng/mL (IQR 0.08 – 0.30 ng/mL). For AKI patients, the respective median

Table 1. Patient characteristics grouped according to the occurrence of acute kidney injury.
Patient demographic data and clinical characteristics of all patients enrolled, grouped according to the development of AKI yes or no. AKI was defined according to the highest RIFLE score attained within 48 hours following admission. RIFLE categories Risk, Injury, and Failure were defined as, respectively, SCr above 150%, 200%, and 300%, of the median of age-specific SCr reference values. Continuous data are expressed as median (interquartile range (IQR)) and categorical data are expressed as number (%). *P-values indicate comparison between AKI- and non-AKI patients using univariate analyses for categorical variables ([a]Pearson's chi-square test or Fisher's exact test, as appropriate) and continuous variables ([b]Mann-Whitney U test). AKI, acute kidney injury; GFR, glomerular filtration rate; ICU, intensive care unit; PIM II, Pediatric index of mortality; PRISM II, Pediatric Risk of Mortality II; RIFLE, risk injury failure loss end-stage renal disease; RSV, *Respiratory syncytial virus*.

	All patients (n=100)	**Non-AKI (n=65, 65%)**	**AKI (n=35, 35%)**	***P*-value***
Baseline Characteristics				
Gender, *male*	66 (66)	42 (65)	24 (69)	0.690[a]
Gestational age, *weeks*	39.0 (37.6 – 40.0)	38.9 (37.8 – 40.0)	39.0 (37.4 – 40.0)	0.861[b]
Birth weight, *kilograms*	3.1 (2.8 – 3.6)	3.1 (2.8 – 3.5)	3.1 (2.8 – 3.6)	0.989[b]
Clinical Characteristics at intubation				
Age, *days*	27.7 (1.5 – 85.5)	27.1 (1.8 – 71.4)	30.3 (1.4 – 115.0)	0.667[b]
Weight, *kilograms*	3.8 (3.2 – 5.2)	3.7 (3.3 – 5.0)	3.8 (3.1- 6.0)	0.745[b]
Admission diagnosis				
Congenital diaphragmatic hernia	*23 (23)*	*16 (25)*	*7 (20)*	*0.132[a]*
Respiratory failure	*20 (20)*	*11 (17)*	*9 (26)*	
Cardiac failure	*18 (18)*	*8 (12)*	*10 (28)*	
RSV Bronchiolitis	*17 (17)*	*14 (21)*	*3 (9)*	
Sepsis	*14 (14)*	*9 (14)*	*5 (14)*	
Other	*8 (8)*	*7 (11)*	*1 (3)*	
Cardiac arrest on ICU admission, yes	10 (10)	5 (8)	5 (14)	0.283[a]
Severity of illness at ICU admission				
PIM II, %	*9.8 (3.4 – 18.8)*	*7.0 (1.7 – 12.0)*	*15.9 (8.4 – 38.1)*	***0.011[b]***
PRISM II, %	*33.8 (11.0 – 64.5)*	*25.7 (10.1 – 54.1)*	*48.9 (25.5 – 77.1)*	***<0.001[b]***
Type of mechanical ventilation				
Pressure control	*68 (68)*	*45 (69)*	*23 (66)*	*0.671[a]*
Pressure regulated volume control	*19 (19)*	*11 (17)*	*8 (23)*	
High frequency ventilation	*11 (11)*	*7 (11)*	*4 (11)*	
Pressure support	*2 (2)*	*2 (3)*	-	
Fraction of inspired oxygen at intubation, *percentage*	59 (40 – 94)	55 (40 – 90)	68 (39 – 100)	0.422[b]
Need for Nitric Oxide ventilation at intubation, *yes*	18 (18)	10 (15)	8 (23)	0.354[a]
Need for two or more vasopressors at intubation, *yes*	49 (49)	24 (37)	25 (71)	***0.001[a]***
Diuretic drugs, *yes*	77 (76)	46 (71)	30 (86)	0.095[b]
Aminoglycosides, *yes*	37 (37)	24 (36)	13 (37)	0.983[b]
Outcomes				
Need for renal replacement therapy, *yes*	1 (1)	-	1 (3)	*N.A.*
Duration of mechanical ventilation, *days*	5.8 (3.1 – 11.4)	4.4 (3.0 – 8.3)	8.3 (5.6 – 19.1)	***0.001[b]***
Length of ICU stay, *days*	10.0 (6.1 – 27.0)	8.3 (5.8 – 15.9)	19.2 (7.8 – 35.6)	***0.002[b]***
Length of hospital stay, *days*	15.9 (8.1 – 38.0)	11.6 (6.5 – 27.9)	27.0 (11.1 – 46.9)	***0.015[b]***
Mortality	20 (20)	9 (14)	11 (32)	***0.027[a]***
ICU	*17*	7	***10***	*N.A.*
Time from admission until death, days	*11.7 (4.4 – 27.2)*	*9.9 (3.4 – 17.0)*	*18.7 (6.7 – 45.0)*	*N.A.*

uNGAL and KIM-1 concentrations were 107 ng/mL (IQR 22.4 – 935) and 0.19 ng/mL (IQR 0.10 – 0.43), significantly higher than for non-AKI patients: uNGAL 23.2 ng/mL (IQR 9.6 – 93) and KIM-1 0.13 ng/mL (IQR 0.08 – 0.25) (both *P*-values <0.001 by Mann-Whitney U tests).

Figure 1 presents the patterns of median uNGAL and KIM-1 levels from 0 to 72 hours following ICU admission. Both patterns show an increase, whereby the mean uNGAL concentration peaked at 6 – 12 hours and the mean KIM-1 concentration at 12 – 24 hours. Urinary NGAL levels were significantly higher in the patients who developed AKI compared to the non-AKI patients at all time frames except for T6 (48 to 72 hours) (all *P*-values ≤0.038, Mann-Whitney U tests). KIM-1 levels were significantly higher in AKI patients only at T2 (12 to 24 hours) and T3 (24 to 36 hours) (both *P*-values ≤0.041 by Mann-Whitney U tests). Table 2 shows the uNGAL and KIM-1 concentrations for T0 (0 to 6 hours), T1 (6 to 12 hours), and T2 (12 to 24 hours) as well as peak levels within 24 hours by SCr-based RIFLE strata. Worse RIFLE status was associated with significantly higher uNGAL and KIM 1 levels (all *P*-values ≤0.018 by Kruskal Wallis tests). Compared across diagnostic categories, urinary NGAL and KIM-1 concentrations were both highest in sepsis patients, especially those who met the AKI criteria (both *P*-values <0.001 by Kruskal-Wallis test) (Figure 2).

uNGAL and KIM-1 and mortality

Seventeen patients died during admission. Three of these non-survivors died within the first 72 hours, of whom one was classified in the non-AKI group (died after 66 hours, peak uNGAL value 114 ng/mL) and two in the AKI-group (died after 46 and 54 hours, both peak uNGAL values 6000 ng/mL). Non-survivors' uNGAL levels at 12 to 24 hours post-admission as well as 24 hours peak levels were higher than those of survivors (*P*-values ≤0.009, Mann-Whitney U tests). There was no significant difference between survivors and non-survivors with regard to KIM-1 levels (all *P*-values >0.05).

ROC analysis

Table 3 shows the AUCs for the prediction of the development of AKI within 48 hours following admission for both biomarkers at T0, T1, and T2 as well as for 24h peak levels. The maximal AUC for uNGAL was 0.815 (95% CI 0.685 - 0.945, *P*-value <0.001) at T0 (0 to 6 hours), with an optimal cut-off value of 126 ng/mL with a sensitivity of 76% and a specificity of 84%. The AUC for uNGAL was 0.780 (95% CI 0.678 - 0.882, *P*-value <0.001) at T1, 0.711 (95% CI 0.599 - 0.824, *P*-value 0.001) at T2, and 0.811 (95% CI 0.719 - 0.902, *P*-value <0.001) for the 24 hour peak levels. KIM-1 was moderately discriminative only at T2 with an AUC of 0.737 (95% CI 0.628 - 0.847, *P*-value <0.001) and an optimal cut-off value of 0.19 ng/mL with a sensitivity of 72% and specificity of 67%. Figure 3 shows the ROC curve of uNGAL and KIM-1 levels at T0, T1, and T2 and the 24 hours peak levels.

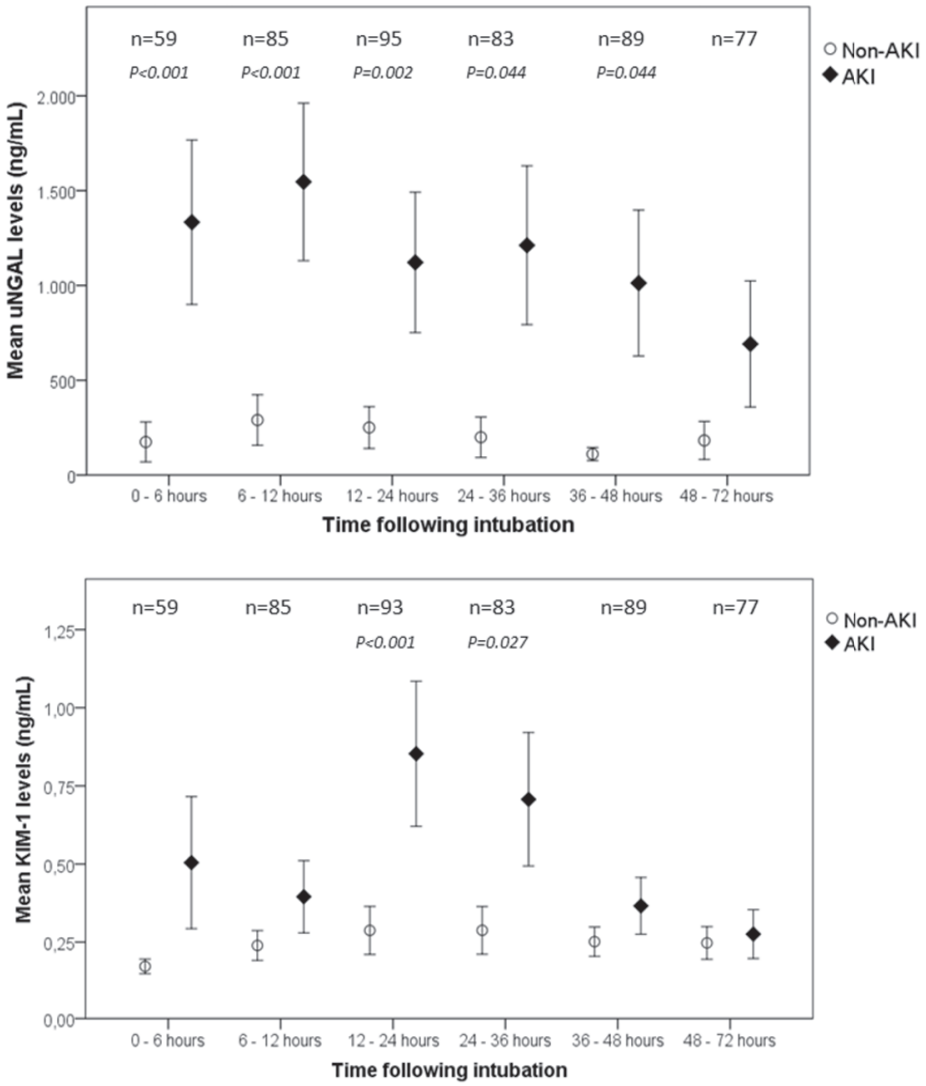

Figure 1. The clinical course of mean urinary NGAL and KIM-1 levels from 0 to 72 hours following intubation, stratified by the occurrence of acute kidney injury within 48 hours post-admission.
Biomarker concentrations are expressed in ng/mL and data represent the mean (± 1 standard error of the mean; SEM). The filled diamonds represent AKI patients whereas the open circles represent Non-AKI patients. Differences were assessed for each biomarker per time-frame using Mann-Whitney U tests. AKI, acute kidney injury; KIM-1, kidney injury molecule-1; uNGAL, urinary neutrophil gelatinase-associated lipocalin;

Table 2. T0, T1, T2 and peak uNGAL and KIM-1 concentrations by SCr-based RIFLE status within 48 hours following admission.
Data are expressed as median (interquartile range (IQR). P-values indicate overall comparison of all groups (that is, Non-AKI versus Risk versus Injury versus Failure). Intergroup differences were assessed using the Kruskal-Wallis test. RIFLE categories Risk, Injury, and Failure were defined as, respectively, SCr above 150%, 200%, and 300%, of the median of age-specific SCr reference values. AKI, acute kidney injury; KIM-1, kidney injury molecule-1; uNGAL, urinary neutrophil gelatinase-associated lipocalin; SCr, serum creatinine.

Measurement, ***number of patients***	**Non-AKI,** ***n=65***	**RIFLE-Risk,** ***n=15***	**RIFLE-Injury,** ***n=10***	**RIFLE-Failure,** ***n=10***	***P*-value**
T0, 0 – 6 h					
uNGAL (ng/mL), *n=59*	31 (16 – 111)	396 (70-1250)	385 (77 – 1987)	1873 (484 – 3936)	***<0.001***
KIM-1 (ng/mL), *n=59*	0.11 (0.08 – 0.19)	0.18 (0.13 – 0.28)	0.10 (0.08 – 0.11)	1.2 (0.4 – 3.5)	***0.004***
T1, 6 – 12 h					
uNGAL (ng/mL), *n=85*	21 (8 – 116)	114 (61 – 420)	275 (11 – 4630)	2430 (727 – 6000)	***<0.001***
KIM-1 (ng/mL), *n=85*	0.11 (0.08 – 0.27)	0.10 (0.08 – 0.29)	0.12 (0.08 – 0.16)	0.35 (0.29 – 1.21)	***0.018***
T2, 12 – 24 h					
uNGAL (ng/mL), *n=95*	22 (10 – 98)	34 (22 – 200)	47 (26 – 1935)	979 (301 – 6000)	***0.001***
KIM-1 (ng/mL), *n=93*	0.16 (0.08 – 0.28)	0.26 (0.11 – 0.56)	0.30 (0.12 – 0.41)	0.47 (0.26 – 2.15)	***0.002***
24h Peak level					
uNGAL (ng/mL), n=*100*	59 (16 – 136)	225 (89 – 730)	385 (56 – 3938)	1495 (387 – 6000)	***<0.001***
KIM-1 (ng/mL), *n=100*	0.17 (0.08 – 0.34)	0.26 (0.11 – 0.56)	0.25 (0.10 – 0.41)	0.86 (0.44 – 2.15)	***0.001***

uNGAL and KIM-1 concentrations preceding AKI

Twenty-four patients met the AKI criteria already at the time of ICU admission, and 19 other patients developed AKI later on within 72 hours; 11 within 48 hours and another eight between 48 and 72 hours post-admission. These 19 patients reached RIFLE-Risk or higher at a median of 34 hours (IQR 20 – 53) post-admission (Risk n=16, 84%; Injury n=3, 16%). For the analysis of biomarkers preceding AKI, the time point at which AKI first occurred was recoded to T=0. All available uNGAL and KIM-1 measurements preceding this time point were recoded relative to T=0 (Figure 4). Using the optimal cut-off value for uNGAL (126 ng/ml) and KIM-1 levels (0.19 ng/mL) as described above, uNGAL was the most sensitive biomarker to predict development of AKI, i.e. in 16/19 (84%) cases versus 11/19 (58%) cases using KIM-1 concentration.

The maximum biomarker levels in urine samples preceding AKI (T=0) were then used to evaluate the diagnostic performances of both biomarkers for AKI prediction. The first urine samples of 49 patients who did not develop AKI served as controls. The clinical characteristics and outcomes of these 49 control patients did not differ from those of the 19 AKI-patients (all *P*-values >0.05). Maximum uNGAL and KIM-1 levels were observed at a median of, respectively, 22 hours (IQR 12 – 24) and 9 hours (IQR 5 – 15) before reaching RIFLE-Risk or higher for the first time. A contingency table analysis using a cut-off value of uNGAL>126 ng/mL showed that the sensitivity of uNGAL was 84%, the specificity

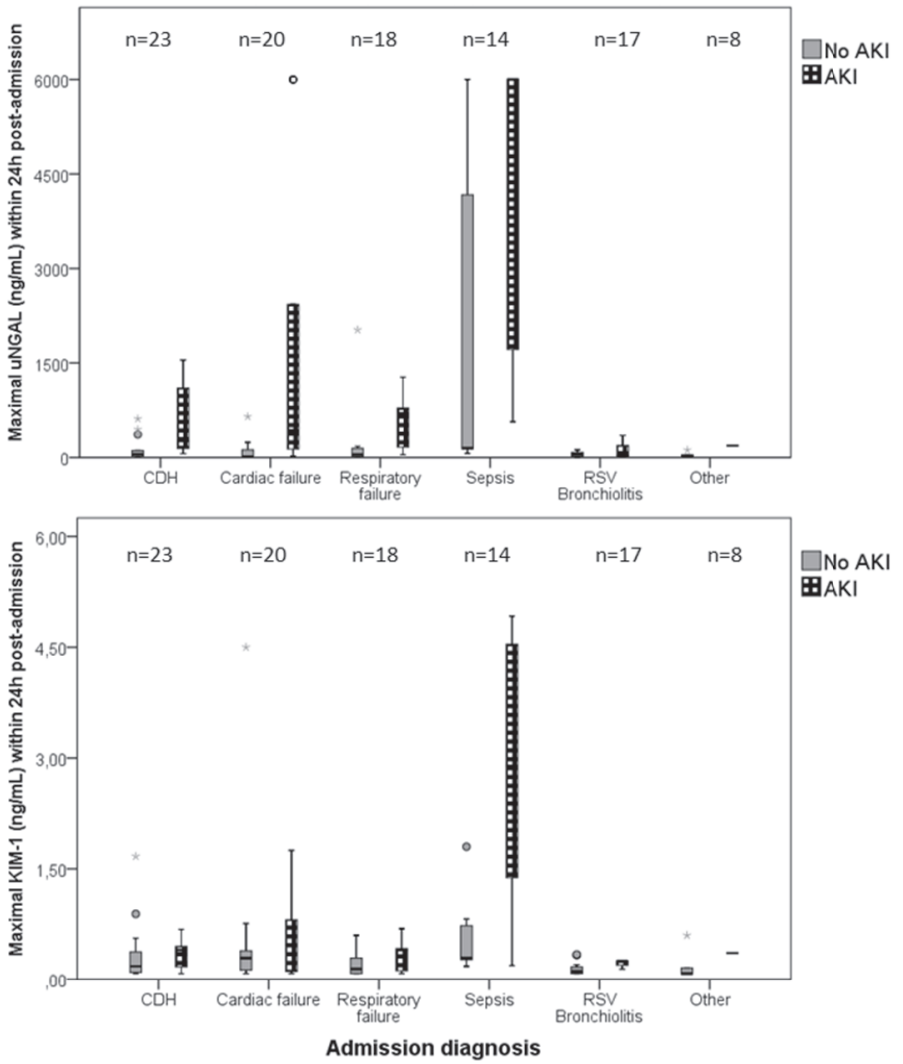

Figure 2. Maximal urinary NGAL and KIM-1 levels within 24 hours post-admission stratified by diagntosis on admission (Total number of patients = 101).
Biomarker concentrations are expressed in ng/mL and data represent the mean (± 1 standard error of the mean; SEM). The black-white boxes represent AKI patients whereas the gray boxes represent Non-AKI patients. Intergroup differences were assessed using Kruskal-Wallis tests. KIM-1, kidney injury molecule-1; uNGAL, urinary neutrophil gelatinase-associated lipocalin;

86%, the positive predictive value 70%, and the negative predictive value 93% (Figure 5). At a cut-off value of 0.19 ng/mL, the sensitivity of KIM-1 was 58%, the specificity 78%, the positive predictive value 50%, and the negative predictive value 83%.

Table 3. Receiver operating characteristic area under the curve for the occurrence of AKI within 48 hours following intubation, using time-frames T0, T1, T2 as well as peak levels obtained within 24h post-admission. The optimal cut-off was based on the Youden index. AUC, area under the curve; CI, confidence interval; KIM-1, kidney injury molecule-1; uNGAL, urinary neutrophil gelatinase-associated lipocalin.

Biomarker	Number of patients, n (%)	Time-frame	AUC (95% CI)	*P-value*	*Cut-off (ng/mL)*	*Sensitivity (%)*	*Specificity (%)*
uNGAL	*59 (59%)*	*T0, 0 – 6h*	0.815 (0.685 - 0.945)	***<0.001***	*126*	*76*	*84*
	85 (85%)	*T1, 6 – 12h*	0.780 (0.678 - 0.882)	***<0.001***	*88*	*70*	*74*
	95 (95%)	*T2, 12 – 24h*	0.711 (0.599 - 0.824)	***0.001***	*32*	*72*	*62*
	100 (100%)	*24h Peak level*	0.811 (0.719 - 0.902)	***<0.001***	*1338*	*80*	*77*
KIM-1	*58 (57.4%)*	*T0, 0 – 6h*	0.618 (0.469 - 0.768)	0.135	0.15	52	60
	85 (85%)	*T1, 6 – 12h*	0.553 (0.469 - 0.729)	0.135	0.13	55	60
	93 (931%)	*T2, 12 – 24h*	0.737 (0.628 - 0.847)	***<0.001***	*0.19*	*72*	*67*
	100 (100%)	*24h Peak level*	0.695 (0.584 - 0.807)	***0.001***	*0.24*	*71*	*62*

Persistent AKI

Of the 24 patients who had AKI on admission, 13 had 'persistent AKI' at time-frame 48 – 72 hours whereas in 11 the RIFLE score improved. Biomarker levels did not significantly differ between both groups.

DISCUSSION

This study aimed to characterize uNGAL and KIM-1 patterns throughout the first days of ICU admission in critically ill children requiring mechanical ventilation and to assess their properties for identifying children developing AKI. We have shown that levels of both biomarkers increased following admission, and were significantly higher with worsening AKI severity. Most importantly, we found that uNGAL levels in the first 6 hours following admission can serve as a marker for identifying children meeting AKI-criteria within 48 hours of ICU admission. Besides, when using the optimal cut-off uNGAL value (126 ng/mL), 16 out of 19 patients in whom AKI was detected 24 hours later would have been diagnosed correctly. In contrast, KIM-1 was not found reliable to identify children at risk for AKI. Thus, in a heterogeneous group of critically ill children, uNGAL may allow for an early diagnosis of AKI, even before a rise in SCr becomes apparent.

Of the 35 patients diagnosed with AKI within 48 hours following admission, a remarkable proportion of almost 70% already had AKI on admission, including the most severe cases. These figures are in line with a large retrospective cohort study evaluating 2106 admissions to the pediatric ICU where AKI predominantly occurred on the first PICU day (2).

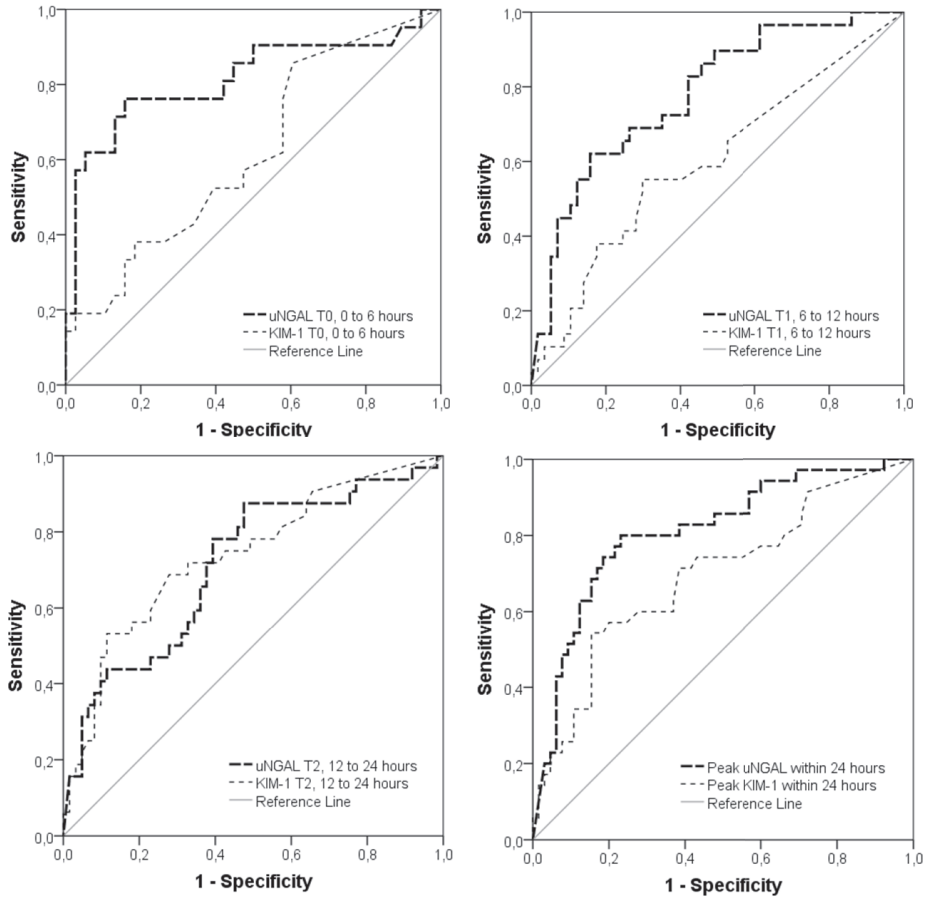

Figure 3. Receiver-operating characteristics curve of urinary NGAL and KIM-1 levels at time-frames T0, T1, T2 and for the 24 hours peak levels.
The black line represents the receiver-operation characteristics curve of uNGAL whereas the dotted line represents the curve of KIM-1. The grey line represents the reference line. KIM-1, kidney injury molecule-1; uNGAL, urinary neutrophil gelatinase-associated lipocalin;

Overall we found notably higher uNGAL and KIM-1 biomarker levels than reported previously (27, 28). Zappitelli and colleagues reported a median peak uNGAL level of 55 ng/mL (IQR 105) for patients with RIFLE-F, while we found a median value of 1495 ng/mL (IQR 387 – 6000) (28). This is even more surprising seeing that 23% of patients in that study had sepsis compared to only 14% in our study. Ours as well as other studies have shown that patients with sepsis have the highest levels of uNGAL and KIM-1, irrespective of AKI development, which is at least in part due to systemic inflammation (25, 31, 35, 36). Another explanation may lie in the lower severity of illness in the Zappitelli-cohort as reflected by a median PRISM II score of 19 (IQR 12) for the renal failure group com-

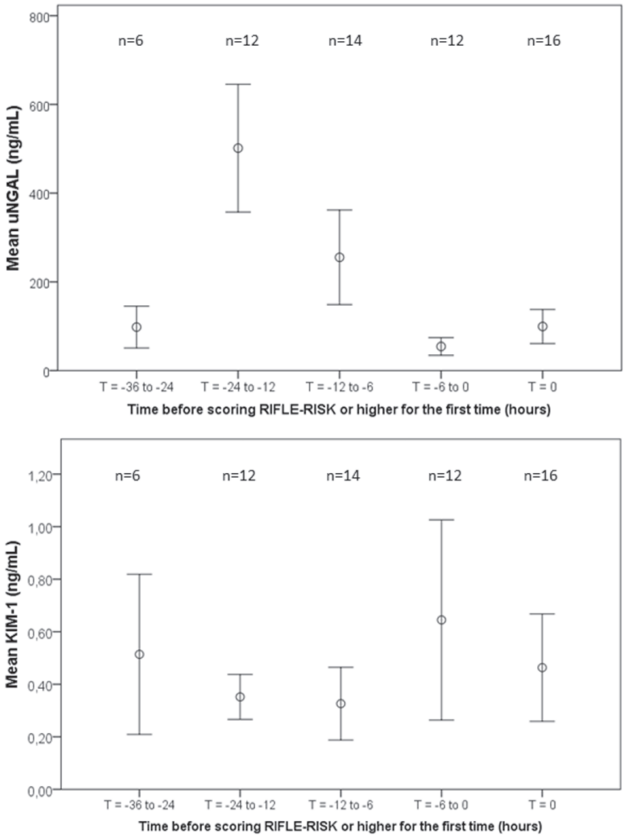

Figure 4. Urinary NGAL and KIM-1 levels prior to AKI onset, defined as attaining RIFLE-Risk or higher (n=19). Biomarker concentrations are expressed in ng/mL and data represent the mean (± 1 standard error of the mean; SEM). KIM-1, kidney injury molecule-1; uNGAL, urinary neutrophil gelatinase-associated lipocalin; n, number

pared with 48.9 (25.5 – 77.1) in our AKI patients. Furthermore, as uNGAL and KIM-1 levels in premature infants and young children generally are higher, the younger age of our subjects might form another explanation (29, 37, 38). Lastly, Zappitelli and colleagues used other biomarker kits (28).

We have shown that levels of both biomarkers increased following admission, with uNGAL levels peaking between 6 and 12 hours and KIM-1 levels peaking somewhat later, between 12 and 24 hours. These patterns resemble those reported in a well-conducted study of 543 adult ICU patients, in which uNGAL levels in AKI patients increased right from the time of admission ($P<0.0001$) and KIM-1 levels first differentiated between non-AKI and AKI 24 hours post-admission ($P=0.008$) (39). To the best of our knowledge there are no pediatric studies available on the patterns of biomarkers following ICU admission using hourly time-intervals. One study in 13 asphyxiated newborns reported uNGAL

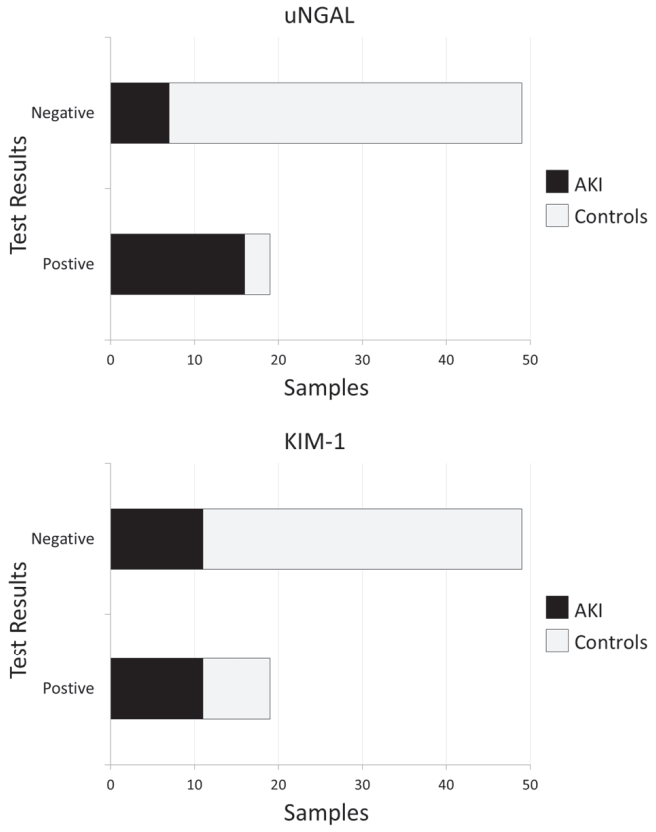

Figure 5. Bar graph showing the results of a contingency analysis
Bar graphs showing the results of a contingency table analysis for uNGAL (cut-off value 126 ng/mL) and KIM-1 (cut-off value of 0.19 ng/mL). A total of 49 control samples were included (non-AKI critically ill), and 19 from critically ill children who developed AKI within 72 hours post-admission. KIM-1, kidney injury molecule-1; uNGAL, urinary neutrophil gelatinase-associated lipocalin

and KIM-1 levels on days 1 and 3 of life (27). Notably, uNGAL levels in this study were increased but remained stable whereas KIM-1 levels were higher only the first day of life, but substantially decreased thereafter (27).

In our study, uNGAL measured within 0 – 6 hours following admission had a reasonable ability (ROC-AUC 0.815) to identify children meeting AKI-criteria within 48 hours (25). The AUC for uNGAL was in perfect line with previous reports in adults (AUCs 0.66 – 0.88, depending on the severity of AKI). The optimal uNGAL cut-off value found (126 ng/mL; sensitivity 75%, specificity 84%) was slightly lower than reported for adults (247 ng/ml; sensitivity 89%, specificity 70% for prediction of renal failure) (40). The discriminative ability of KIM-1, in contrast, is only limited since all AUC were ≤0.73, which is consistent with previous reports (27, 39). All in all, the most robust NGAL results in critically ill chil-

dren come from Mishra and colleagues in children who underwent CPB in whose study AKI timing and etiology post-CPB is well defined (23, 25).

Concerning the time-relationship between the biomarker levels and AKI development, in the present study uNGAL levels peaked at 12 to 24 hours before reaching RIFLE class Risk for the first time while KIM-1 levels preceding AKI remained steady. A similar pattern for uNGAL levels was reported in a study in adults, but in that study KIM-1 level did not rise until the time AKI was diagnosed (39). Zappitelli and colleagues evaluated uNGAL levels relative to day of pRIFLE AKI attainment in 21 pediatric ICU patients (28). Although they presented NGAL levels relative to urine creatinine concentrations, it was clear that median NGAL level peaked at one day before AKI onset (28). Blood was sampled daily, however, instead of hourly intervals.

The false-positive results of uNGAL (14%) and KIM-1 (22%) in the present study may be due to the limited specificity of both biomarkers but one can also speculate that subclinical AKI occurred; a condition in which there is tubular damage without a rise in SCr as a sign of glomerular filtration alteration (10). Still, uNGAL was able to predict AKI development correctly in 16 out of 19 children, which further demonstrates its potential as an early marker of tubular damage. Identifying children in the early stage of AKI (e.g., already at presentation in the emergency room or during clinical deterioration at the general ward) may help develop early interventions to limit the development of AKI. In this light, several studies have been published on potential protective properties of drugs against the development of AKI, including atrial natriuretic factor, e.g. in case of cisplatin treatment (41) and cardiac surgery (42), and administration of bovine derived alkaline phosphatase in critically ill patients with sepsis-associated AKI (43). Even though the rationale behind the renoprotective effects remain to be fully elucidated, these studies illustrate that new options in the prevention or limitation of the severity of AKI are currently being investigated.

Several limitations of this study should be addressed. First, this study has a single-center design, which may limit the generalization of these data to other institutions. Still, since we did not focus on diagnostic subclasses, our cohort can be considered a representative sample of the general pediatric ICU population aged <1 year requiring mechanical ventilation. Second, the overall sample size was too small for multiple subgroup analyses. Besides, since we exclusively used SCr without urine criteria for grading AKI severity, we may have underscored the incidence and grade of AKI (44). Furthermore, we were not able to collect all urine samples planned, especially not during the first hours of admission when an indwelling urine catheter yet had to be placed, the urine portion was needed for clinical purposes e.g. screening for metabolic diseases, or when a patient was anuric. Still 86% of all planned samples were collected. Finally, uNGAL and KIM-1 levels preceding AKI could be analyzed in only a small sample because most AKI patients already met RIFLE-stage Risk or higher on admission.

CONCLUSIONS

This study shows that both uNGAL and KIM-1 levels in critically ill infants with AKI increase following ICU admission and peak, respectively, 6 to 12 hours and 12 to 24 hours thereafter. Notably, of the children who met AKI criteria within 48 hours following admission, almost 70% already had AKI on admission when entering the ICU. Still, uNGAL reliably discriminated between infants who met AKI criteria within 48 hours following admission and those who did not. In addition, uNGAL was able to predict AKI development correctly in 84% children, before any rise in SCr became apparent. These findings support the emerging role of uNGAL in identifying AKI at an early stage, which, in the future, may help us to establish timely renoprotective interventions to reduce AKI in those most vulnerable patients in hospital.

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7 Urinary neutrophil gelatinase-associated lipocalin predicts renal injury following extracorporeal membrane oxygenation

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ABSTRACT

Objective: To evaluate the course of urinary neutrophil gelatinase-associated lipocalin (uNGAL) and kidney injury molecule-1 (uKIM-1) levels in young children during extracorporeal membrane oxygenation (ECMO) and concomitant continuous hemofiltration. Furthermore, to evaluate whether these levels predict outcome.

Design: Prospective observational cohort study from July 2010 to July 2013.

Setting: ICU of a level III university children's hospital

Patients: Thirty-one ECMO-treated children up to one year of age were included.

Interventions: None.

Measurements and Main Results: Patients were weaned from ECMO after a median of 162 hours (IQR 83–304). Throughout the study 58% of the patients met the criteria for acute kidney injury (AKI) (that is, RIFLE-Risk or higher defined as an increase in SCr corresponding to ≥150% when compared to age-specific reference values). Levels of both biomarker patterns changed significantly throughout ECMO (uNGAL *P*-value<0.001 and uKIM-1 *P*-value=0.005, linear mixed model analyses). uNGAL levels were already high before ECMO whereas uKIM-1 levels increased throughout the first ECMO day and peaked at 12 to 24 hours. Also, uNGAL levels at 12 to 24 hours of ECMO therapy were higher among patients with AKI post-ECMO (*P*-value=0.002, Mann-Whitney U tests). Biomarker levels did not differ between survivors and non-survivors.

Conclusions: The increased uNGAL and uKIM-1 levels confirm that renal tubular damage occurs in critically infants in need of ECMO. The fact that the maximal uNGAL levels were measured 24 hours earlier than uKIM-1 supports the use of biomarker combinations rather than a single biomarker to identify patients at risk of AKI. Lastly, since uNGAL levels at 12 to 24 hours of ECMO therapy were associated with AKI post-ECMO, this marker may facilitate more timely adjustment of therapeutic interventions.

INTRODUCTION

Extracorporeal membrane oxygenation (ECMO) is an advanced cardiopulmonary bypass technique applied in acute reversible respiratory- or cardiovascular failure. Children requiring ECMO are at increased risk of acute kidney injury (AKI), which we and others reported in up to two-thirds of children on ECMO without pre-emptive renal replacement therapy (1-5). Moreover, AKI has been associated with poor outcomes including mortality and chronic kidney disease on the longer term (1-8). As such, AKI during ECMO in children remains a major concern.

In most clinics around the world the diagnosis of AKI relies on two functional abnormalities: a relative change in serum creatinine (SCr) levels and/or oliguria. However, SCr is a delayed and unreliable indicator of AKI for several reasons. First, SCr is influenced by non-renal factors such as age and muscle mass (9). Second, SCr does not increase until 25-50% of the nephrons are damaged, but with decline of renal function the percentage of creatinine excretion due to active tubular secretion increases (10-12). Third, directly after birth, a newborns' SCr reflects the maternal level (10). There above, in ECMO-treated patients, SCr concentrations will be diluted on account of the large blood volume in the extracorporeal circuit. Also, when ECMO-treated patients concomitantly receive continuous hemofiltration (CH) – either on indication or pre-emptively – extrarenal SCr elimination further masks early stages of renal injury. Adding CH, however, was found associated with a shorter time to decannulation and fewer ventilator days after ECMO (13, 14). Thus, in young children treated with ECMO, especially those receiving concomitant CH, the shortcomings of traditional markers of kidney damage are even more prominent. This, in turn, hampers the possibility to tailor therapy (e.g., dose adjustment or switch to other drugs) to improve ECMO outcomes (15).

Recent advances in critical care nephrology have resulted in the discovery of novel biomarkers specific for tubular injury rather than for glomerular filtration rate (GFR) such as neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1). NGAL is a 25kDa protein that belongs to the lipocalin superfamily (16, 17). It was first found in activated neutrophils, and later in other cells as well, including liver, lungs and kidney cells (18-20). Following kidney injury, NGAL reabsorption by the proximal tubules may decrease whereas the NGAL expression in the distal nephrons may be strongly upregulated (21). A recent meta-analysis supports the use of NGAL as a biomarker for the prediction of AKI in several clinical settings including cardiac surgery, critical care and transplantation, mainly in adults (22). KIM-1 is a transmembrane protein that contains an immunoglobulin domain and a mucin domain (23). It is expressed in proximal tubule cells and is thought to promote clearance of apoptotic and necrotic cells (24). Following ischemic or toxic kidney injury, KIM-1 is highly upregulated and subsequently shed into the urine and extracellular space (23-25). In a recent well-conducted meta-analysis,

urinary KIM-1 (uKIM-1) had a considerable predictive value for early AKI detection principally in cardiac surgery patients, but its potential value in other clinical settings still needs to be confirmed (26).

A literature search identified three studies on serum and urinary NGAL in ECMO-treated children but none on uKIM-1 in this setting (27-29). One included children with congenital heart disease and found that serum NGAL levels at the first ECMO day were higher in those patients who also received CH (28). The other two studies were pilot studies in which a subset of critically ill children received ECMO. It was found that urinary NGAL (uNGAL) had a high sensitivity to identify children with AKI on admission or within the first 48 hours of illness (27, 29). It is hard to draw a general conclusion from these studies because none reported successive uNGAL levels following cannulation, few ECMO patients were included (maximal n=10) and the patients' age-range was wide – from one week up to 21 years. Clearly, a knowledge gap remains in our understanding of the utility of renal tubular injury biomarkers in children receiving ECMO support and pre-emptive CH. The aim of our study was, therefore, to evaluate the course of uNGAL and uKIM-1 in young children treated with ECMO and pre-emptive CH. Secondly we aimed to evaluate whether these biomarker levels are related to adverse outcomes including AKI after ECMO decannulation and ICU mortality.

MATERIALS AND METHODS

Setting

This prospective observational cohort study was performed from July 2010 to July 2013 at the ICU of the Erasmus MC – Sophia Children's Hospital in Rotterdam, The Netherlands. This level III ICU is one of the two designated centers providing pediatric ECMO in The Netherlands. ECMO treatment in our ICU is available since 1992, and is most often performed in newborns and young children.

Patients

Children (born ≥37 week of gestational age) up to the age of one year who received ECMO-support in combination with pre-emptive CH were eligible for inclusion. The following exclusion criteria were applied: (1) congenital anomalies of the kidney or urinary tract (CAKUT), (2) inclusion impossible for logistical reasons, or (3) parents refused consent. If a patient underwent more than one ECMO run, only data related to the first run were used. The study protocol was approved by the local medical ethical review board. Deferred consent was used in combination with written informed consent obtained from the primary caregivers within 12 hours following ECMO cannulation. Urine and

blood samples collected for the purpose of this study from children for whom eventually written informed consent was refused were destroyed.

Standard of clinical care

Our clinical ICU guidelines support ECMO-treatment to be offered to patients suffering from potentially reversible cardio-respiratory diseases unresponsive to conventional intensive care and whose risk of death is estimated to be greater than 80% using the criteria of Stolar and colleagues (30, 31). The ECMO circuit was primed as described elsewhere (14). From 2005 onward, all patients received by protocol pre-emptive continuous hemofiltration (CH) by placement of a hemofilter (Multiflow 60; Hospal, Lyon, France) parallel to the ECMO circuit and distal to the roller pump. The CH predilution flow rate of the filtration fluid (HF-BIC32, Dirinco, Rosmalen, The Netherlands) was at the default of 50 mL/kg/hour. Ultrafiltration was targeted to maintain a daily neutral or negative fluid balance depending on the patient's clinical condition. During the study period, institutional policy regarding ECMO support including the use of pre-emptive CH did not change.

Sample collection and analytical procedures

Blood and urine samples for AKI markers were collected concomitantly at the following time-points: prior to ECMO, during ECMO at 0-6 hours, 6 – 12 hours, 12 – 24 hours, 24 – 36 hours, 36 – 48 hours, 48 – 72 hours, 72 – 96 hours, 96 – 120 hours, and 120 – 144 hours. Blood samples only were also collected on the first three days post-ECMO. Blood samples were drawn from an indwelling arterial catheter. Urine samples were collected from a routinely placed bladder catheter. In order to obtain fresh samples, the urine collection bag was emptied one hour prior to each sample time-point. Collected urine samples were left refrigerated for sedimentation for a period of 1 to 2 hours, aliquoted and stored within four hours after collection at -80^{o}C until the assay. At some time-points, urine could not be collected due to anuria or logistical problems.

All biomarker measurements were performed in the Erasmus MC department of Clinical Chemistry. Creatinine was determined by an enzymatic assay (Creatinine Plus; Roche Diagnostics, Banchburg, NG) on a Cobas 8000 analyzer. During the period of sample collection, the interassaycoefficient of variation was less than 2.6%.

uNGAL was measured using the uNGAL immunoassay developed for a standardized clinical platform (ARCHITECT analyzer, Abbott Diagnostics Division, Abbott Laboratories, Abbott Park, IL, USA). The mean inter-assay coefficient of variation for uNGAL was 5.3% at a concentration of 19.4 ng/mL. The limit of quantification (LoQ) of NGAL was 3.0 ng/mL. The reagents and calibrator for the uNGAL assays were kindly supplied by Abbott Diagnostics (Abbott Laboratories, Abbott Park, IL, USA).

uKIM-1 was measured using a commercially available enzyme-linked immunosorbent assay kit (ELISA) (BioAssay Works, Ijamsville, MD, USA). The mean inter-assay coefficient of variation for uKIM-1 was <14% at a concentration of 0.17 ng/mL. The LoQ of uKIM-1 was 0.08 ng/mL.

Data collection

For each patient the usual demographic data were collected (e.g., sex, gestational age, birth weight) as well as clinical characteristics related to the ICU course including primary diagnosis leading to the initiation of ECMO as well as type of mechanical ventilation, FiO_2 need post-intubation, need for vasopressor drugs and/or nitric oxide (NO) ventilation. Furthermore, age and body weight at ECMO cannulation was recorded as well as Severity of illness and organ dysfunction at ICU admission was assessed with the Pediatric Risk of Mortality III (PRISM III) score and the Pediatric Index of Mortality II (PIM II) (32, 33). Other data collected were: type of ECMO (venoarterial (VA), venovenous (VV) and/or conversion), major abdominal or thoracic surgery, cardiopulmonary resuscitation, ECMO duration, prolonged renal replacement therapy post-ECMO, days on mechanical ventilation, length of stay in our ICU, and ICU mortality. With regard to mortality we distinguished between "early deaths", when a child could not be weaned from ECMO, and "late deaths", when a child survived ECMO decannulation but nevertheless died in the ICU.

Definitions

AKI was classified according to the maximum SCr-based RIFLE score obtained either 24 hours pre-ECMO or within 36 hours post-ECMO. As all children received CH during ECMO, it was not possible to reliably assess AKI during ECMO using the SCr-based RIFLE score. The RIFLE classification defines three grades of increasing AKI severity: Risk (Risk for kidney injury), Injury (Injury to the kidney) and Failure (Failure of kidney function) (34). RIFLE outcome categories L (Loss of renal function) and E (End-stage renal disease) were not applicable, as the study was restricted to 36 hours following ECMO decannulation. Since for most children SCr baseline concentrations pre-ICU admission were not available or the SCr values still reflected maternal levels, RIFLE scores were calculated from actual SCr concentrations compared to the 50^{th} percentile of reference values for age (35). An SCr concentration above 150% of the median SCr reference value indicated RIFLE category Risk, above 200% category Injury, and above 300% category Failure. These SCr reference values were obtained from children without kidney disease, by using small age intervals ranging from 1 day in the first week after birth up to 3 months at the end of the first year of age (35).

Data analysis

All data are presented as median values with interquartile range (IQR) for continuous variables or numbers with percentages (%) for categorical variables, unless indicated otherwise. uNGAL and uKIM-1 concentrations were expressed as a ratio of urinary creatinine (uCr) concentration (ng/mg uCr). The concentrations were than compared to age- and gender specific reference values (36). Biomarker concentrations were also compared between patients who received VV versus VA ECMO by using Mann-Whitney U tests. Associations between biomarker levels at two time-points following cannulation (that is, 0 to 12 hours and 12 to 24 hours) and renal outcome post-ECMO (that is, non-AKI versus AKI) as well as mortality (that is, late death versus survival) were explored in univariate comparisons using Mann-Whitney U tests. McNemar's test was used to explore the relation between individual RIFLE strata pre-ECMO versus post-ECMO.

Linear mixed models were used to describe the longitudinal changes in the uNGAL and uKIM-1 concentrations. The dependent variables in these linear mixed models were log-transformed values of the uNGAL and uKIM-1 concentrations, and the independent variable was the time-point, coded as a categorical variable. uNGAL and uKIM-1 concentrations have a very skewed distribution, and the log-transformation of the dependent variable was necessary to ensure that the model residuals were approximately normally distributed. A random intercept was used in the linear mixed models to account for the within-subject correlations. Median uNGAL and uKIM-1 levels and 95% CIs of the median levels were calculated for each time-point by applying the exponential function to the estimated marginal means (i.e. means corrected for covariates) and associated 95% CIs of the log-transformed uNGAL and uKIM-1 values.

To test whether the biomarker patterns changed significantly during the time on ECMO, F test for the variable 'time-point' were performed in linear mixed models that were estimated without the pre-ECMO observations. Two-sided P=0.05 was considered the limit of significance in all analyses. P-values were presented without correction for multiple testing. Data were analyzed using IBM SPSS statistics version 20 (Statistical Package for the Social Sciences, Chicago, IL).

RESULTS

From July 2010 to July 2013, 47 children met the eligibility criteria and were considered for inclusion. Six were excluded since they suffered from CAKUT, four due to logistical problems, and six because parents did not provide written informed consent. Thus, data of 31 children were analyzed (Figure 1).

Fifteen (48%) were male, and at start of ECMO median age was 5.6 days (IQR 2.2 – 36.7) and body weight 3.4 kilograms (IQR 3.0 – 4.5). The most common primary diagnoses ne-

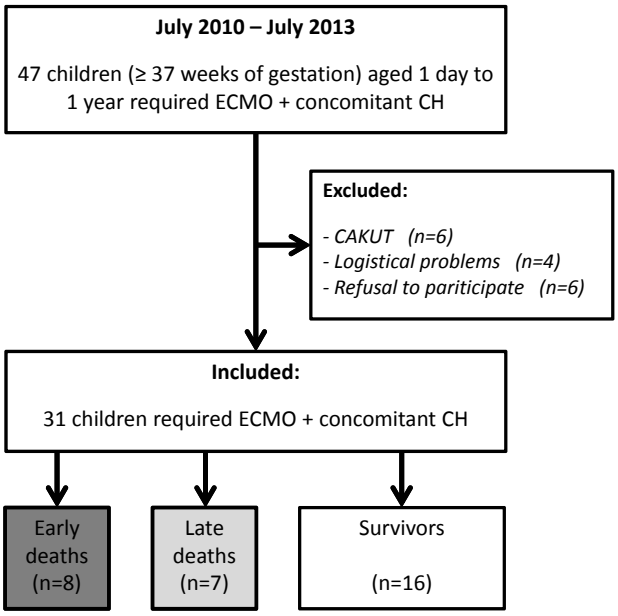

Figure 1. Patient inclusion flowchart.
Flowchart detailing inclusion and exclusion criteria for infants aged 1 day to 1 year who were treated with ECMO and concomitant CH, which resulted in the final study cohort. With regard to mortality we distinguished between "early deaths", when a child could not be weaned from ECMO, and "late deaths", when a child survived ECMO decannulation but nevertheless died in the ICU. CAKUT, congenital anomalies of the kidney and urinary tract; ICU, intensive care unit; NS, non-survivors; S, survivors

cessitating ECMO were congenital diaphragmatic hernia (CDH) (n=10; 32%), meconium aspiration syndrome (MAS) (n=5; 16%), and isolated persistent pulmonary hypertension (n=5; 16%). Table 1 provides detailed patient characteristics.

Thirteen children (42%) initially received VV ECMO, which in 2 (15%) was converted to VA ECMO due to ongoing hemodynamic instability unresponsive to fluid resuscitation and medication. Patients were weaned from ECMO after a median of 162 hours (IQR 83 – 304). Overall mortality was 15/31 (48%) and there were eight "early deaths" (53%) (failure to wean ECMO) – after a median of 8.3 days (IQR 4.3 - 13.5) of ECMO support – versus seven "late deaths" (47%) – at a median of 4.4 days (IQR 3.7 – 6.3) after ECMO decannulation. For seven of the "early deaths" (88%) the underlying diagnosis comprised a major congenital anomaly which was incompatible with life e.g., inoperable hypoplastic left heart syndrome, alveo-capillary dysfunction or CDH.

Table 1. Patient characteristics

Table 1 shows demographic data and clinical characteristics of all patients enrolled. Continuous data are expressed as median (interquartile range (IQR)) and categorical data are expressed as number (%).With regard to mortality we distinguished between "early deaths", when a child could not be weaned from ECMO, and "late deaths", when a child survived ECMO decannulation but nevertheless died in the ICU. ICU, intensive care unit; n, number; PPHN, persistent pulmonary hypertension; VA, venoarterial; VV, venovenous.

Baseline Characteristics	**All patients (n=31)**
Gender, *male*	15 (48)
Ethnicity, *Caucasian*	21 (68)
Gestational age, *weeks*	38.6 (37.6 – 39.9)
Birth weight, *kilograms*	3.0 (2.7 – 3.5)
At ECMO cannulation	
Postnatal age, *days*	5.6 (2.2 – 36.7)
Weight, *kilograms*	3.4 (3.0 – 4.5)
Primary diagnosis	
Congenital diaphragmatic hernia	*10 (32)*
Meconium aspiration syndrome	*5 (16)*
Isolated PPHN	*5 (16)*
Respiratory failure	*4 (13)*
Cardiac failure	*4 (13)*
Sepsis	*3 (10)*
Severity of illness at ICU admission	
PIM II, %	*15 (7 – 27)*
PRISMII, %	*28 (22 – 37)*
Ventilator Settings	
Ventilator Mode	
Pressure control	20 (65)
High frequency ventilation	9 (29)
Other	2 (6)
Amount of oxygen needed, percentage	*96 (61 – 100)*
Nitric oxide ventilation, yes	*20 (65)*
Need for vasopressors, *yes*	27 (87)
Time from ICU admission until start ECMO, *days*	2.2 (0.4 – 7.8)
During ECMO	
ECMO mode, *venovenous*	13 (42)
Conversion VV – VA	2 (7)
Major Surgery, *yes*	12 (39)
Cardiopulmonary Resuscitation, *yes*	4 (13)
ECMO duration, *hours*	162 (83 – 304)
Post-ECMO	
Need for prolonged renal replacement therapy, *yes*	2 (6)
Duration of mechanical ventilation, *days*	13.8 (6.3 – 27.1)
Length of ICU stay, *days*	14.3 (7.0 – 59.5)
Mortality	
ICU	*15 (48)*
"Early deaths"	*8 (53)*
Major congenital anomaly incompatible with life	*7 (87)*

AKI evolution

Thirteen patients (42%) met the criteria for AKI within the 24 hours prior to ECMO. Seven (23%) were classified as Risk, 3 (10%) as Injury, and 3 (10%) as Failure but none received renal replacement therapy before ECMO (Figure 2). All 13 received CH by protocol starting at the initiation of ECMO. Despite CH and thus extra-renal SCr elimination, 11 patients still had maximum SCr concentrations during ECMO indicating AKI (Risk n=4, 36%; Injury n=6, 55%; Failure n=1, 10%).

Renal outcome post-ECMO was evaluated only for the 23 patients who were successfully weaned from ECMO. Eight met the criteria for AKI post-ECMO (Risk n=2, 25%; Injury n=1, 13%; Failure n=5, 63%). Two of those received renal replacement therapy; one died on post-ECMO day 3 and the other patient survived until ICU discharge. Figure 2 shows the AKI evolution from prior-ECMO cannulation to post-ECMO. Only thirteen patients (42%) never had AKI at any point. There was no relation between AKI pre-ECMO versus post-ECMO (*P*-value >0.05, McNemar'stest).

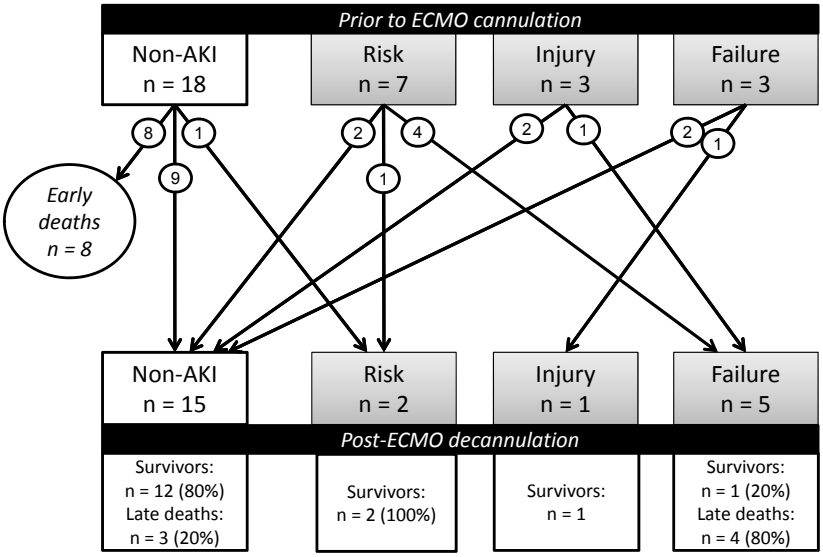

Figure 2. The evolution of acute kidney injury based on serum creatinine levels compared to age-specific reference values, assessed prior to ECMO and after ECMO.

AKI was defined according to the highest RIFLE score attained prior to- and post-ECMO. RIFLE categories Risk, Injury, and Failure were defined as, respectively, SCr above 150%, 200%, and 300%, of the median of age-specific SCr reference values. With regard to mortality we distinguished between "early deaths", when a child could not be weaned from ECMO, and "late deaths", when a child survived ECMO decannulation but nevertheless died in the ICU. ICU, intensive care unit; NS, non-survivors; S, survivors

Urinary biomarker patterns

For all 31 patients, the median number of samples collected per patient was 6 (IQR 3 – 8). For these patients the median uNGAL and uKIM-1 concentrations during ECMO were 598 ng/mg uCr (188 – 1702) and 0.39 ng/mg uCr (0.24 – 0.93), respectively. Biomarker concentrations and SCr concentrations relative to age-specific reference values are presented per time-point in the Supplemental Digital Content 1. Biomarker concentrations at any of the time-points did not significantly differ between children who initially received VV ECMO versus those who received VA ECMO (*P*-value >0.05, Mann-Whitney U tests).

Figure 3 shows the temporal course of median NGAL and uKIM1 levels from 12 hours before ECMO to 144 hours following ECMO cannulation, according to the linear mixed model analyses. Significant changes were found for both biomarker patterns during

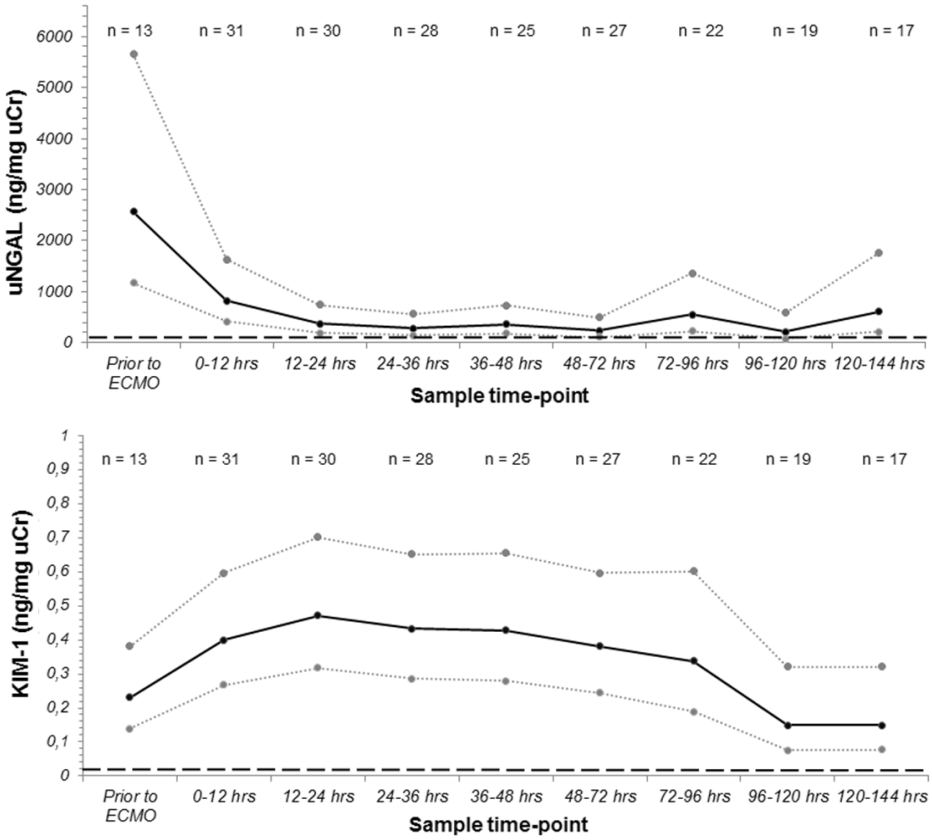

Figure 3. The temporal course of (a) uNGAL (ng/mg uCr) and (b) uKIM-1 (ng/mg uCr) levels according to a linear mixed model analysis.
The black line represents the median value, the dotted lines demarcate the 95% confidence intervals. The black dashed line represents the age-specific reference values. Hrs, hours; uCr, urinary creatinine; uKIM-1, urinary kidney injury molecule-1; uNGAL, urinary neutrophil gelatinase-associated lipocalin

ECMO (uNGAL *P*-value <0.001 and uKIM-1 *P*-value =0.005). Within the first 12 hours of ECMO treatment, uNGAL levels dropped sharply from median 2570 ng/mg uCr (95% CI 1170 – 5648) to 827 ng/mg uCr (95% CI 418 – 1637) (*P*-value <0.001). Despite the decrease, all patients still had uNGAL levels above the median of uNGAL reference values specific for age- and gender and 84% (n=26 patients) above the 95th percentile. Hereafter uNGAL levels decreased more slowly. uKIM-1 levels increased throughout the first ECMO day from median 0.40 ng/mg uCr (95% CI 0.27 – 0.60) to 0.47 ng/mg uCr (95% CI 0.32 – 0.70) at 12 to 24 hours. At both time-points, all patients except one (97%) had uKIM-1 levels above the 95th percentile of uKIM-1 reference values (36). Similar to the pattern of uNGAL, uKIM-1 levels steadily decreased after this peak and remained stable throughout the subsequent ECMO course.

Adverse outcomes

Relations between uNGAL and uKIM-1 levels and adverse outcomes were evaluated at: 0 to 12 hours and 12 to 24 hours following cannulation given that both biomarkers peaked at these time-points.

Table 2. (a) uNGAL (ng/mg uCr) and (b) KIM-1 (ng/mg uCr) concentrations within 24 hours following ECMO cannulation related to survival and outcome.
Acute kidney injury was defined according to the highest RIFLE score attained within 36 hours following ECMO decannulation. RIFLE categories Risk, Injury, and Failure were defined as, respectively, SCr above 150%, 200%, and 300%, of the median of age-specific SCr reference values. uCr, urinary creatinine; uKIM-1, urinary kidney injury molecule-1; uNGAL, urinary neutrophil gelatinase-associated lipocalin; SCr, serum creatinine.

Table 2a NGAL (ng/mg uCr)	**Survival**			**AKI post-ECMO**		
Sample time-point during ECMO	Survivors (n=16)	Late deaths (n=7)	*P*-Value	Non-AKI following ECMO (n=15)	AKI following ECMO (n=8)	*P*-Value
0 – 12 hours	619 (238 – 4447)	896 (51 – 2614)	0.721	383 (51 – 3621)	2550 (560 – 7546)	0.114
12 – 24 hours	127 (69 – 780)	350 (76 – 11775)	0.368	**80 (64 – 297)**	**1235 (170 – 2472)**	***0.002***

Table 2b KIM-1 (ng/mg uCr)	**Survival**			**AKI post-ECMO**		
Sample time-point during ECMO	Survivors (n=16)	Late deaths (n=7)	*P-Value*	Non-AKI following ECMO (n=15)	AKI following ECMO (n=8)	*P-Value*
0 – 12 hours	0.39 (0.14 – 0.45)	0.34 (0.25 – 0.58)	0.879	0.31 (0.17 – 0.45)	0.40 (0.23 – 0.58)	0.167
12 – 24 hours	0.27 (0.12 – 0.89)	0.78 (0.43 – 1.69)	0.127	0.41 (0.14 – 1.1)	0.42 (0.29 – 2.5)	0.545

At neither time-point did uNGAL levels and uKIM-1 levels significantly differ between survivors and non-survivors (all *P*-values >0.05, Mann-Whitney U tests) (Table 2). At 12 to 24 hours, uNGAL levels in post-ECMO AKI patients were higher than in non-AKI patients (*P*-value = 0.002, Mann-Whitney U tests), but levels did not differ at 0 to 12 hours. At neither time-point did uKIM-1 levels differ between post-ECMO AKI patients and non-AKI patients.

DISCUSSION

Our results show that in a group of patients, in whom the traditional markers SCr and urinary output are not reliable, renal tubular injury markers uNGAL and uKIM-1 strongly fluctuate throughout ECMO support and concomitant CH. Patterns differed, however. NGAL urinary levels were already increased before the start of ECMO treatment, and decreased following ECMO cannulation but were still far above reference levels for age (36). In contrast, uKIM-1 levels started to increase 24 hours later. Hence, this study supports the notion that using biomarker combinations rather than one single biomarker has added value for identifying patients at risk for AKI. Despite the relatively small patient sample, we were able to demonstrate that uNGAL levels at 24 hours post-ECMO cannulation were related to AKI diagnosis post-ECMO.

Before ECMO cannulation, AKI was present in as many as 13 patients (42%). During ECMO, still 11 patients (35%) had AKI according to the SCr-based RIFLE criteria, in spite of concomitantly received CH, which provides extra-renal creatinine clearance of maximally 15 ml/min. Notably, eight of the eighteen patients pre-ECMO classified as non-AKI could not be weaned from ECMO ("early deaths"). One could speculate that these were the ones who received "early onset ECMO" to treat organ failure (alveo-capillary dysfunction or CDH). Survivors, on the other hand, mostly suffered from systemic conditions (e.g., sepsis) and had already been exposed to numerous risk factors for AKI prior to the start of ECMO including persistent hypotension unresponsive to IV fluids and vasopressors or nephrotoxic drugs.

Another important finding of this study concerns the evolution of AKI throughout ECMO. Eventually, 18 of the 31 patients (58%) had any rise in SCr concentrations indicating AKI (defined as RIFLE-Risk or higher) at some point prior to or after ECMO-treatment. This high percentage is in line with previous studies reporting on AKI incidences in severely ill children admitted to an ICU (1-5).

For all 31 patients median uNGAL and uKIM-1 concentrations during ECMO were fairly high – indicating substantial tubular damage – although lower than uNGAL/uCr concentrations reported in previous studies (29). This may be due to the fact that previous studies mainly enrolled children with septic shock, whereas sepsis was diagnosed in

only 10% of our ECMO subjects. Sepsis is a common inducer of AKI and rapidly induces massive upregulation of NGAL expression in both serum and urine (37, 38). Another explanation could be the use of different types of biomarker assays across all studies.

In our study neither uNGAL nor uKIM-1 levels differed between survivors and non-survivors. In 39 critically ill children with septic shock (of whom 10 received ECMO) Wai and colleagues found a lower median uNGAL/uCr concentration in survivors than in non-survivors (29). Eighty percent of our non-survivors however, suffered from major congenital anomalies that were incompatible with life, rather than sepsis and associated multiple-organ failure. uNGAL levels in these patients may therefore not adequately predict risk of death. Moreover, since our sample size was even smaller than the Wai study, This may potentially obscure a relationship between uNGAL and mortality.

Krawczeski and colleagues were the first to evaluate urinary NGAL and KIM-1 as predictors of post-cardiac surgery AKI (39). Their data show that uNGAL levels were significantly increased at 2 hours while uKIM-1 significantly increased only at 12 hours post-surgery (39). They also found that biomarker combinations improved prediction of AKI beyond clinical models (39). This temporal relationship of uNGAL and uKIM-1 following the initiation of ECMO is quite similar to that found in our study, with uKIM-1 increasing also 12 hour later than uNGAL post-cannulation, and peaking approximately 24 hours later than uNGAL. The finding that uNGAL levels were increased explicitly before the start of ECMO suggests that a renal insult occurs early in the course of critical illness. The decline in uNGAL levels following cannulation, in turn, may reflect an improved circulation and tissue oxygenation due to the ECMO circuit. One may speculate, however, that a so-called "renal stress state" may persist in most patients given that uNGAL and uKIM-1 levels were above the 95th percentile of age- and gender specific reference values at most time-point during ECMO.

In our study uKIM-1 peak-levels during ECMO were not related to AKI post-ECMO, which may in part be due to the relatively small sample size, but also to the heterogeneity of our patient group in terms of underlying diagnosis (22, 26). Still, uNGAL levels at 24 hours post-ECMO cannulation were associated with AKI post-ECMO and, as such, this biomarker is promising for the early identification of AKI patients, who may benefit from more timely and targeted renoprotective interventions.

Several limitations of this study should be addressed. First, this was a single-center analysis with a relative small sample size. Second, we did not have extensive data on the ICU stay pre-ECMO or post-ECMO. This is unfortunate since many patients might already have developed AKI preceding ECMO. As we are a tertiary referral center, most transferred patients were placed on ECMO within hours following admission. Moreover, they were transferred back to the referring facility when their clinical condition was improved and stable. Third, AKI was defined by the SCr-based RIFLE classification, which has inherent limitations to accurately estimate GFR. Moreover, we did not include urine output in the

RIFLE criteria which can help in the staging of AKI in critically ill patients (40) – because this was influenced by the amount of fluid removed by concomitant CH.

CONCLUSIONS

Our study is, to the best of our knowledge, the first to establish a temporal course of uNGAL and uKIM1 in critically infants in need of ECMO and on CH, in whom the shortcomings of the traditional markers of kidney damage (e.g., SCr levels, urinary output) are even more prominent. Seeing that both biomarkers at most time-points greatly exceeded age- and gender specific reference values, our study confirms that renal tubular damage occurs in this population. Furthermore, the maximal uNGAL levels were measured 24 hours earlier than uKIM-1, which further supports the use of biomarker combinations rather than a single biomarker to identify patients at risk of AKI. Lastly, timely adjustment of therapeutic interventions are possible with the use of uNGAL because its level at 24 hours post-cannulation was associated with the occurrence of AKI post-ECMO.

Supplemental Digital Content 1

uNGAL (ng/mg uCr), uKIM-1 (ng/mg uCr) and serum creatinine levels for each sample time-point. Serum creatinine levels at each time-point were compared to baseline concentrations were compared to the 50th percentile of age-corrected reference values (35). Creatinine was determined by an enzymatic assay (Creatinine Plus; Roche Diagnostics, Banchburg, NG) on a Cobas 8000 analyzer. uNGAL was measured using the uNGAL immunoassay developed for a standardized clinical platform (ARCHITECT analyzer, Abbott Diagnostics Division, Abbott Laboratories, Abbott Park, IL, USA). KIM-1 was measured using a commercially available enzyme-linked immunosorbent assay kit (ELISA) (BioAssay Works, Ijamsville, MD, USA). Data are presented as median and interquartile range. uCr, urinary creatinine; uKIM-1, urinary kidney injury molecule-1; uNGAL, urinary neutrophil gelatinase-associated lipocalin; SCr, serum creatinine

	Number of patients with urine samples available	uNGAL (ng/mg uCr)	uKIM-1 (ng/mg uCr)	SCr compared to age-specific reference values (%)
Prior to ECMO	n=13	576 (191 – 6507)	0.13 (0.07 – 0.26)	112 (97 – 133)
During ECMO				
0 – 12 hours	n=31	528 (164 – 3575)	0.27 (0.17 – 0.44)	95 (81 – 123)
12 – 24 hours	n=30	247 (71 – 818)	0.45 (0.26 – 0.95)	104 (81 – 133)
24 – 36 hours	n=28	244 (81 – 458)	0.47 (0.24 – 0.87)	97 (74 – 135)
36 – 48 hours	n=25	323 (71 – 1229)	0.28 (0.16 – 0.70)	96 (77 – 121)
48 – 72 hours	n=27	159 (82 – 400)	0.38 (0.16 – 61)	92 (75 – 133)
72 – 96 hours	n=22	779 (109 – 2356)	0.23 (0.16 – 0.49)	103 (83 – 136)
96 – 120 hours	n=19	127 (108 – 238)	0.14 (0.13 – 0.24)	98 (78 – 143)
120 – 144 hours	n=17	258 (204 – 716)	0.17 (0.12 – 0.22)	94 (80 – 137)

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8 Urinary NGAL, FGF-2, and EGF concentrations in critically ill neonates suffering from acute kidney injury: a prospective observational study

BACKGROUND

Acute kidney injury (AKI) (previously named acute renal failure) is generally characterized by the abrupt inability of the kidneys to adequately excrete waste products and regulate fluid and electrolyte homeostasis (1). In the setting of pediatric critical care AKI represents a common and serious clinical problem and is associated with an increased duration of mechanical ventilation and overall stay in the intensive care unit (ICU) with an increased mortality (2, 3).

In current clinical practice serum creatinine (SCr) is used to diagnose AKI although this marker of functional glomerular filtration has several important limitations. First, non-renal factors such as age, gender, diet, and muscle mass may affect SCr levels, especially in young children (4). Also, SCr levels may remain normal until 25 – 50% of functioning nephrons have been lost, whereas at poor renal functioning active tubular creatinine secretion will overestimate actual glomerular filtration (1, 5, 6). Ultimately, neonates SCr levels in the first days of life largely reflects maternal creatinine levels (5). Altogether it is increasingly accepted that SCr is a delayed and relatively insensitive marker of AKI, which shortcomings have driven the search for novel renal injury biomarkers (7). Thus, AKI is detected rather late based on relative changes in SCr while therapeutic intervention might be needed in the early onset of AKI to reverse AKI or to prevent secondary injury from for example nephrotoxic drugs (8). Hence, new urinary biomarkers are evidently needed to timely identify children with AKI and to monitor renal recovery.

Among the candidate biomarkers, neutrophil gelatinase-associated lipocalin (NGAL) has emerged as one of the most promising biomarkers for the early detection of AKI. NGAL is a small 25 kDa protein produced by activated neutrophils and it is also expressed in other human tissues including the lungs, spleen, and healthy kidneys (9-11). In the kidney, NGAL is mainly expressed in epithelial cells of the loop of Henle and distal tubule segments and upon renal tubular injury is strongly up regulated and excreted in the urine (12). In a landmark paper, Mishra and colleagues showed that urine NGAL levels in children at 2 hours post-cardiopulmonary bypass had a nearly 100% accuracy for predicting the occurrence of AKI at 24 – 72 hours (12). Subsequent studies confirmed these findings in adults and evaluated the biomarker performances in different clinical situations including the setting of critical care but did not report results as robust as Mishra did (13-15). This is not surprising since ICU patients generally represent a heterogeneous group of critically ill patients with various disease severities in whom sepsis is commonly encountered, especially in adults. One must realize, however, that NGAL is also produced by normal distal tubular cells and extra-renal tissues, and is highly induced in inflammatory conditions (10, 16). Thus, even though NGAL seems a sensitive and early marker of AKI following cardiopulmonary bypass, this biomarker may not

reliably identify AKI patients in a sepsis cohort, because of the systemic inflammatory nature of the disease.

Recently, two pilot studies done at Children's National Medical Center in Washington, DC, USA, explored the value of urinary NGAL, fibroblast growth factor 2 (FGF-2), and epidermal growth factor (EGF) to assess the outcome of AKI in critically ill children and newborns (17, 18). Both FGF and EGF, were selected because they are involved in the pathogenesis of AKI and the regeneration of renal tubules (18–23). In fact, FGF-2 is released by injured endothelial cells, and renal endothelial injury plays a key role in the pathogenesis of AKI (24). Moreover, the urinary levels of EGF are decreased in children and adults with acute or chronic renal tubular injury (21, 22, 25–28). In the first pilot study, Hoffman et al. followed the outcome of neonates treated with ECMO or hypothermia for hypoxic ischemic encephalopathy, and reported that low EGF levels post-recovery predicted AKI with 73% sensitivity and 82% specificity (17). The second pilot was conducted by Wai and colleagues and evaluated children with septic shock and children receiving ECMO support (18). It was shown that the urinary biomarker profile comprised of NGAL, FGF-2, and EGF, increased the specificity to detect AKI in these children, when compared to each individual biomarker value. Finally, both NGAL and FGF-2 appeared to be good candidates for predicting the risk of death in these critically ill children (18). Even though both pilot studies were well conducted, they focused on children admitted to an ICU with specific conditions, including sepsis, and infants requiring ECMO or therapeutic hypothermia. To the best of our knowledge, data on children who are critically ill due to a variety of underlying diagnosis -not necessarily related to systemic inflammation- are lacking so far. The aim of the current study was therefore, to determine the concentration of FGF-2 and EGF in the urine of critically ill infants, all suffering from AKI secondary to different events not necessarily related to systemic inflammation. Secondly, to define the value of these factors compared with urinary NGAL as the "silver" standard for the diagnosis of severe AKI.

METHODS

Setting

From June 2010 until July 2012 a single-center prospective observational cohort study was conducted in the level III ICU of the Erasmus Medical Center – Sophia Children's Hospital, Rotterdam, The Netherlands. Considered for study enrolment were critically ill children (>37 weeks of gestational age up to one year of age) who had a compromised renal function on ICU entrance, which improved within seven days, following admission. Critical illness was defined as the need for mechanical ventilation with endotracheal

intubation and/or vasopressor drugs. Improvement of renal function was evaluated daily and defined as a decrease in SCr of ≥0.3 mg/dL.

Patients were not eligible for inclusion when they had (1) congenital abnormalities of the kidney or urinary tract, (2) death was anticipated within 24 hours, or (3) received mechanical ventilation for other reasons than pulmonary insufficiency, or (4) required ECMO treatment upon admission. If a patient was admitted to the ICU more than once, only data related to the admission in which the study was performed were included.

Sample collection and analytical procedures

Blood- and urine samples were collected in all children on ICU entrance and every 12 to 24 hours thereafter. Blood samples were drawn from an indwelling arterial line, if available, or by capillary or venous puncture. Urine samples were collected using a bladder catheter or an inlay diaper. In order to obtain fresh samples, the urine collection bag was emptied one hour prior to each sampling time frame. Collected urine samples were left refrigerated and aliquoted and stored within four hours after collection at -80°C in 1mL cryo-tubes and shipped on dry ice to Children's National Medical Center, Washington DC, USA.

Upon arrival in Children's, each sample was centrifuged for 5 minutes at 5000 rounds per minute at 4 degrees Celsius, aliquotted in the correct amounts (volume: 200 uL per vial) and yet again stored at -80°C. For each urinary biomarker, i.e. FGF-2, NGAL, and EGF an Enzyme-linked immunoabsorbent assays (ELISA) procedure was performed according to the manufacturer's instructions (Table 1). All biomarker levels were expressed per milligram urinary creatinine (uCr). Serum creatinine concentrations were measured in the hospital's clinical chemical laboratory by an enzymatic assay (Creatinine Plus; Roche Diagnostics, Banchburg, NG) on a Cobas 8000 analyzer. During the period of sample collection, the interassay coefficient of variation (CV) was less than 2.6%.

Table 1. Characteristics of the enzyme-linked immunosorbent assays used for each biomarker.
EGF, epidermal growth factor; FGF-2, urinary fibroblast growth factor 2; NGAL, urinary neutrophil gelatinase associated lipocalin

Biomarkers	Manufacturer	Limit for detection	Wavelength Microplate reader
NGAL	BioPorto Diagnostics, Denmark	Lower limit for detection: 0.2 ng/mL Upper limit for detection: 20 ng/mL	450nm & 620nm
FGF-2	R&D Systems, Minneapolis, USA	Lower limit for detection: 0.313 pg/mL Upper limit for detection: 20 pg/mL	490nm & 690nm
EGF	R&D Systems, Minneapolis, USA	Lower limit for detection: 3.9 pg/mL Upper limit for detection: 125 pg/mL	450nm & 540nm

Sample selection

For the purpose of the study only two urine samples per patient were selected, based on SCr levels. The first sample, which will be referred to as "before", is the one collected immediately following ICU admission. The second, which will be referred to as "after" was collected subsequent to the onset of renal recovery. Renal recovery was defined as a decrease in SCr of ≥0.3 mg/dL.

Data collection

Data were retrieved from our electronic data registry and 'Patient Data Management System' (PDMS), which stores all prospectively collected physiological parameters, lab results and therapeutic modalities. Demographic variables were retrieved from the patients' medical records: gestational age, birth weight (kilogram), gender, ethnicity, primary diagnosis leading to ICU admission, Apgar scores at 1, 5, and 10 minutes after birth and age on admission (days). The Pediatric Risk of Mortality II (PRISM II) score and Pediatric Index of Mortality II (PIM II) score were collected as an indication of severity of illness (19, 20). The following data during ICU admission were retrieved: SCr concentrations, urinary output (millilitre per kilogram per hour), type of mechanical ventilation together with the fraction of inspired oxygen at the time of intubation, the need for nitric oxide ventilation, length of mechanical ventilation (days), length of ICU stay (days), and survival until ICU discharge. Furthermore, we recorded the administration of vasopressor drugs, diuretics (furosemide or bumetanide) or aminoglycosides (gentamicin, tobramycin or amikacine).

Acute kidney injury

To determine the severity of AKI, patients were assigned RIFLE scores following admission (21). The RIFLE classification defines three grades of increasing AKI severity, including category 'R' (Risk for kidney injury), category 'I' (Injury to the kidney), and category 'F' (Failure of kidney function) (21). RIFLE outcome categories L (Loss of renal function) and E (End-stage renal disease) were not applicable, as the study was restricted to the first seven days following ICU admission. Since in many cases no pre-admission SCr concentration was available RIFLE categories R, I, and F were defined as SCr concentrations above 150%, 200%, and 300%, respectively, of the median age-specific SCr reference value (22). These SCr reference values were obtained from children without kidney disease, using small age intervals ranging from 1 day in the first week after birth up to 3 months at the end of the first year of age. When renal replacement therapy was provided, SCr measurements were disregarded.

Ethical research board

The study was approved by the Medical Ethical Research Board of the Erasmus Medical Center – Sophia Children's Hospital (MEC-2010-070).

Statistical analysis

Data were analysed using Statistical Package for the Social Sciences (SPSS) ver. 21 (SPSS, Chicago, IL). Data are presented as median and Inter Quartile Range (IQR) unless indicated otherwise. To obtain RIFLE scores, SCr values were expressed in percentages of the median of age-specific reference values. Biomarker levels were compared between survivors and non-survivors using univariate analyses for continuous variables (Mann-Whitney U test). Two-sided P=0.05 was considered the limit of significance in all analyses. Wilcoxon Signed Rank test was used to compare biomarker concentrations in the "before" versus "after" samples. Using the "before" samples, receiver operating characteristics curve (ROC) curves were generated for the occurrence of severe AKI (Risk or Injury-Failure). The area under the curve (AUC) with 95% confidence intervals (95% CI), were calculated. Also, for each time frame the optimal cut-off level based on the Youden index was calculated with corresponding sensitivity and specificity.

RESULTS

The study cohort consisted of 20 critically ill patients of whom 15 (75%) survived until ICU discharge. Table 2 details the characteristics for all patients. The median age and weight on admission were 2.7 days (IQR 0.7 – 68.7 days) and 3.8 kilogram (IQR 3.1 – 4.1 kilogram) respectively. The most common primary diagnoses that were reason to initiate mechanical ventilation were sepsis (n=7, 35% of all patients), congenital diaphragmatic hernia (n=4, 20% of all patients), and congenital heart disease (n=4, 20% of all patients). Pressure control ventilation was the preferred type of mechanical ventilation (n=15, 75% of all patients) with a median fraction of inspired oxygen of 52%, and combined with nitric oxide ventilation in 5 patients. Throughout the subsequent ICU course 16 patients (80%) received two or more vasopressor drugs while 17 patients (85%) received diuretic drugs.

Renal function

AKI was categorized as Risk in 11 cases, as Injury in 6 cases, and as Failure in 3 cases (55%, 30%, and 15% of all patients, respectively). At the end of the study period, only 2 patients were still classified as having AKI (both in the Risk category) whereas all other patients (18 patients) had recovered. The median time until the onset of recovery was 5.6 days (IQR 4.3 – 6.2). The median urine output (milliliter per kilogram per hour) was 2.2 mL/kg/hour (IQR 0.6 – 3.6) over the first day following the start of mechanical ventilation, which output increased to 5.8 mL/kg/hour (IQR 3.2 – 7.9) at the end of the study period.

Table 2. Detailed patient characteristics.
Table 2 shows patient demographic data and clinical characteristics of all patients enrolled. Continuous data are expressed as median (interquartile range (IQR)) and categorical data are expressed as number (%). ICU, intensive care unit, PIM II, Paediatric Index of Mortality; PRISMII, Paediatric Risk of Mortality Score III.

Baseline characteristics	**Critically ill patients n=20**
Gender, *Female*	7 (35)
Ethnicity, *Caucasian*	16 (80)
Gestational Age, *weeks*	38.9 (37.0 - 40.0)
Birth weight, *kilograms*	3.0 (2.8 - 3.7)
Apgar score	
1 minute after birth	*7 (5 - 8)*
5 minutes after birth	*8 (6 - 9)*
10 minutes after birth	*8 (8 - 9)*
ICU admission characteristics	
Age on admission (days)	2.7 (0.7 - 68.7)
Weight on admission (kilograms)	3.8 (3.1 – 4.1)
Diagnosis	
Sepsis	*7 (35)*
Congenital diaphragmatic hernia	*4 (20)*
Congenital heart disease	*4 (20)*
Pulmonary disease	*3 (15)*
Non-congenital heart disease	*2 (10)*
Severity of illness at ICU admission	
PIM II, %	*14 (6 – 23)*
PRISM II, %	*39 (21 – 61)*
Type of mechanical ventilation	
Pressure control	*15 (75)*
Pressure regulated volume control	*3 (15)*
High frequency ventilation	*2 (10)*
Fraction of inspired oxygen at intubation, *percentage*	52 (39 – 90)
Need for nitric oxide ventilation at intubation, *yes*	5 (25)
Need for two or more vasopressors at intubation, *yes*	16 (80)
Diuretic drugs, *yes*	17 (85)
Aminoglycosides, *yes*	10 (50)
Outcomes	
Need for renal replacement therapy, *yes*	1 (5)
Length of mechanical ventilation, *days*	9.0 (6.0 - 18.2)
Length of ICU stay, *days*	24.5 (12.5 - 49.8)
ICU mortality	5 (25)

Biomarker concentrations

For all 20 patients the median urinary biomarker concentrations in the "before" samples were 1081 ng/mg uCr (IQR 324 – 6990) for NGAL, 69 pg/mg uCr (IQR 46 – 146) for FGF-2, and 58964 pg/mg uCr (IQR 28798 – 110264) for EGF. For all 20 patients the median urinary biomarker concentrations in the "after" samples were 338 ng/mg uCr (IQR 75 – 1239) for NGAL, 43 pg/mg uCr (IQR 21 – 66) for FGF-2, and 66071 pg/mg uCr (IQR 41725 – 102232) for EGF. There were no differences in biomarker concentrations between survivors and non-survivors at any time-point (Mann-Whitney U tests, all P-values >0.05).

Comparison of the urinary biomarker concentrations in the "before" samples with those in the "after" samples showed that only FGF-2 and NGAL concentrations significantly decreased over time (Related-Samples Wilcoxon Signed Rank test P-values: 0.028 and 0.014 respectively) (Figure 1). In contrast, urinary levels of EGF were not significantly different between both time-points.

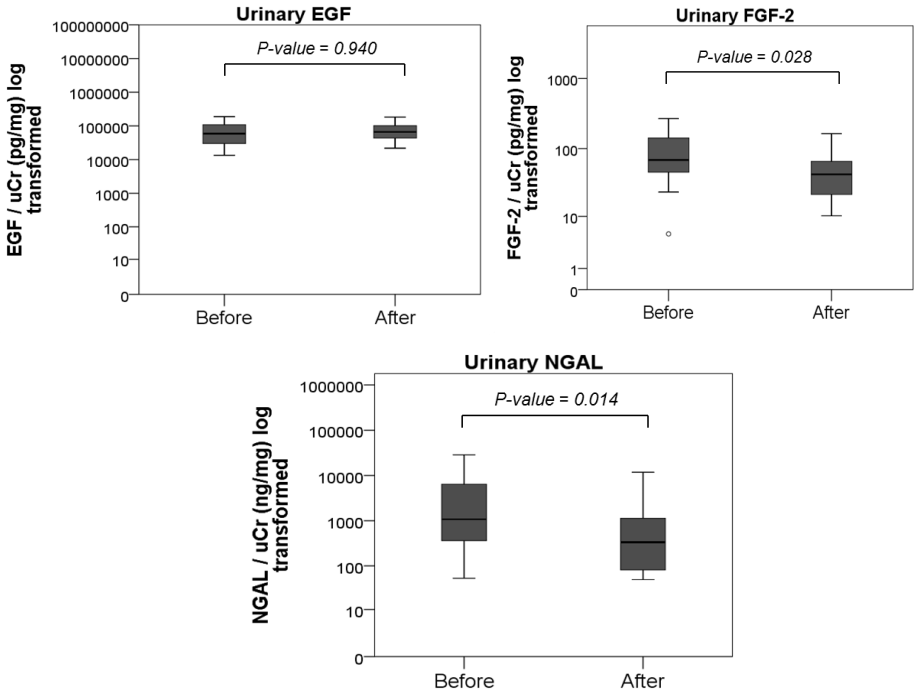

Figure 1. Biomarker concentrations corrected for urinary creatinine in the "before" samples (following ICU admission) compared to the "after" samples (onset of renal recovery).
Plots show median and interquartile range log transformed values. P-values indicate comparison between before and after samples using Related-Samples Wilcoxon Signed Rank test. EGF, epidermal growth factor; FGF-2, urinary fibroblast growth factor 2; NGAL, urinary neutrophil gelatinase associated lipocalin; uCr, urine creatinine.

Receiver operating characteristics curves

Table 3 shows the AUCs for the prediction of severe AKI (RIFLE-Injury or RIFLE-Failure) within seven days following ICU admission. The AUC for NGAL was 0.82 with an optimal cut-off value of 967 ng/mg uCr with a sensitivity of 89% and a specificity of 73%. The AUC for FGF-2 was 0.68 with an optimal cut-off value of 69 pg/mg uCr with a sensitivity of 67% and a specificity of 64%. The AUC for EGF was 0.54 with an optimal cut-off value of 62537 pg/mg uCr with a sensitivity of 56% and a specificity of 64% (Figure 2).

Table 3. Area under the curve of all "before" biomarker concentrations for the occurrence of severe AKI (RIFLE Risk or Injury-Failure) following ICU admission.
AUC, area under the curve; EGF, epidermal growth factor; FGF-2, urinary fibroblast growth factor 2; NGAL, urinary neutrophil gelatinase associated lipocalin; uCr, urine creatinine.

Biomarker	AUC (95% CI)	Cut-off value	Sensitivity	Specificity
NGAL	0.82 (0.62 – 1.00)	967 ng/mg uCr	89%	73%
FGF-2	0.68 (0.43 – 0.92)	69 pg/mg uCr	67%	64%
EGF	0.54 (0.27 – 0.80)	62537 pg/mg uCr	56%	64%

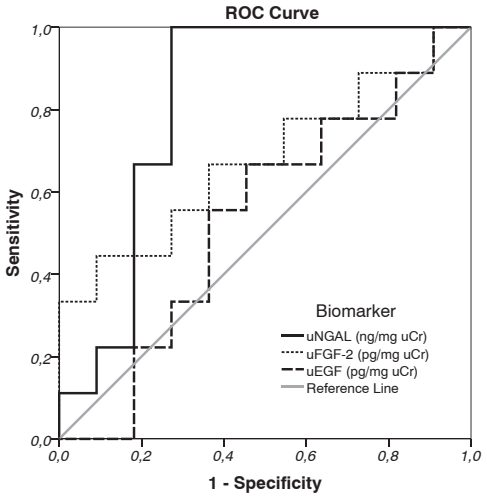

Figure 2. Receiver operating characteristic curve of all "before" biomarker concentrations for the occurrence of severe AKI (RIFLE Risk or Injury-Failure) following ICU admission.
The optimal cut-off was based on the Youden index. AUC, area under the curve; EGF, epidermal growth factor; FGF-2, urinary fibroblast growth factor 2; uNGAL, urinary neutrophil gelatinase associated lipocalin; uCr, urine creatinine.

INTERPRETATION OF RESULTS

This study aimed to evaluate the urinary concentration of FGF-2 and EGF, which are released by injured and regenerating renal endothelial and tubular epithelial cells, in critically ill infants suffering from AKI. The secondary aim was to define the value of these factors compared with urinary NGAL for diagnosing of severe AKI.

Our rationale to select these biomarkers was based on previous studies conducted by Soler Garcia and colleagues, who identified a novel urinary biomarker profile to monitor the outcome of children with HIV-associated nephropathy (HIVAN) (23, 24). More specifically, they found that the urinary levels of the angiogenic growth factor FGF-2 were significantly elevated in correlation with the development of tubular microcysts and renal endothelial injury (23, 24). These changes were also associated with decreased urinary EGF levels, which is a powerful growth factor for renal epithelial cells (23-25). Considering that children with HIVAN show some tubular features that mimic the renal lesions seen in children with AKI, including tubular dilatation and casts, as well as necrosis and sloughing of renal tubular epithelial cells into the lumen, we hypothesized the these candidate biomarkers could be clinically relevant to follow the outcome of critically ill children with AKI. Therefore, we explored their clinical value to assess the outcome of AKI in critically ill children followed in our institution.

Of the 20 critically ill children with AKI enrolled in our study, 11 were classified as Risk, 6 as Injury and 3 as Failure. Urinary NGAL, FGF-2, and EGF concentrations observed in our study are perfect in line with concentrations reported for neonates receiving therapeutic hypothermia or ECMO (17). Somewhat higher urinary NGAL, FGF-2, and EGF concentrations were reported by Wai and colleagues (29). The difference might be explained by the underlying etiology of AKI since Wai and colleagues enrolled children with septic shock only while in our study sepsis was diagnosed in only 7 (35%) of our patients. Sepsis, in turn, has been recognized as a common inducer of AKI and induces rapid up-regulation of NGAL expression in both serum and urine (26, 27).

Following ICU admission, urinary NGAL and FGF-2 significantly decreased within 4 to 7 days. Renal recovery biomarker uEGF, however, did not change over this time period. One explanation might be the degree of recovery. In fact, we collected our so-called "after" sample following the onset of recovery, which was defined as a decrease in SCr concentration of ≥0.3 mg/dL within 4 to 7 days following ICU admission. Obviously, this classification does not at all categorize the degree of recovery. The suggestion that EGF might need a certain time-window before any rise can be observed is further emphasized by the study of Hoffman and colleagues. They studied neonates receiving therapeutic hypothermia and measured urinary biomarker levels at baseline, after 48 hours of illness, and at >24 hours post-recovery (17). They found significantly increased EGF/uCr concentrations in the samples collected at >24 hours post-recovery (17). On the other

hand, this finding was not reproduced in their subset of ECMO patients, which suggests that even 24 hours post-recovery is still too short to detect regeneration biomarkers for recovery. One could also speculate that the underlying etiology of AKI and thus repair mechanisms may differ among hypothermia patients versus ECMO-patients and for sure with a group of critically ill children requiring mechanical ventilation as enrolled within study.

The results of our study suggest that NGAL measured following admission has a reasonable ability (AUC 0.82) to identify children with severe AKI, either RIFLE-Injury or RIFLE-Failure. Neither FGF-2 (AUC 0.68) nor EGF (AUC 0.54) concentrations on admission could reliably identify children with severe AKI. These results are somewhat different from results reported in neonates treated with ECMO or hypothermia. In these patients is was demonstrated that EGF, expressed as ng/ml of urine, had a reasonable ability to identify critically ill neonates with AKI with an AUC of 0.73 (17). On the other hand, no significant differences were detected in the urinary levels of NGAL and FGF-2 between neonates with or without AKI who were treated in a similar manner (17). Hence, the role of FGF-2 as a marker of renal endothelial injury is not fully established by the current study. Although EGF did not perform well to diagnose severe AKI, this biomarker should be re-evaluated in the appropriate research setting where renal recovery is evaluated at multiple pre-defined time-point throughout a patients clinical course; at least at ICU- and hospital discharge.

CONCLUSIONS

Urinary NGAL and FGF-2 significantly decreased within 4 to 7 days following ICU admission. Renal recovery biomarker EGF, however, did not change over this time period. Finally, especially NGAL appeared a good candidate for diagnosing severe AKI. Nonetheless, considering the small sample size, multi-centered cohort studies are needed to validate these results.

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PART IV

PROGRESSION TO CHRONIC KIDNEY DISEASE

9 CKD and hypertension during long-term follow-up in children and adolescents previously treated with extracorporeal membrane oxygenation

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ABSTRACT

Background and objectives: Many children receiving extracorporeal membrane oxygenation develop AKI. If AKI leads to permanent nephron loss, it may increase the risk of developing CKD. The prevalence of CKD and hypertension and its predictive factors during long-term follow-up of children and adolescents previously treated with neonatal extracorporeal membrane oxygenation were determined.

Design, setting, participants, and measurements: Between November of 2010 and February of 2014, neonatal survivors of extracorporeal membrane oxygenation who visited the prospective follow-up program at 1, 2, 5, 8, 12 and 18 years of age were screened for CKD and hypertension (BP ≥95th percentile of reference values). CKD was suspected in children with either an eGFR <90 ml / min per 1.73m^2 or proteinuria (urinary protein-to-creatinine ratio >0.50 for children ages ≤24 months and >0.20 at ages >24 months). The RIFLE classification (risk, injury, or failure as 150%, 200% or 300% of serum creatinine reference values) was used to define AKI during extracorporeal membrane oxygenation without pre-emptive hemofiltration.

Results: Median follow-up of the 169 screened participants was 8.2 years (interquartile range=5.2-12.1 years). Nine children had a lower eGFR but all rates were >60 mL/min/1.73m^2. Proteinuria was observed in 20 children (median=0.26 mg protein / mg creatinine; interquartile range=0.23-0.32 mg protein / mg creatinine), and 32 children had hypertension. Only history of AKI was associated with CKD (*P*=0.004). Children with RIFLE-scores injury and failure had 4.3 times higher odds of CKD signs or hypertension than those without AKI (95% confidence interval, 1.6 to 12.1; *P*=0.004)

Conclusions: Altogether, 54 participants (32%) had at least one sign of CKD and / or hypertension. However, most values were marginally abnormal, with no immediate consequences for clinical care. Nevertheless, a prevalence of 32% clearly indicates that survivors of neonatal extracorporeal membrane oxygenation, especially those with AKI, are at risk of a more rapid decline of kidney function with increasing age. Therefore, screening for CKD development in adulthood is recommended.

INTRODUCTION

AKI is a frequent complication among critically ill children admitted to an intensive care unit (ICU). Reported incidences vary widely, between 4.5% and 82%, depending on the definition used and the population studied (1-6). Moreover, AKI is an independent risk factor for prolonged mechanical ventilation and pediatric ICU mortality (1, 4). Recently, our group and other research groups reported high incidences of AKI in children treated with extracorporeal membrane oxygenation (ECMO) who tend to be the most critically ill admitted to an ICU (7-10).

Severe AKI can result in a substantial loss of functioning nephrons which may lead to hyperfiltration in the spared ones (11). Single-nephron hyperfiltration, in turn, leads to glomerular sclerosis, interstitial fibrosis, and tubular atrophy. These pathologic changes may eventually lead to CKD, a condition characterized by a progressive decline in GFR over time, proteinuria, and / or systemic hypertension (12). Timely therapeutic interventions can slow the progression of renal injury, and medication should be titrated to actual clearance (13, 14).

Over the past years, research has largely focused on short-term implications of AKI. The few follow-up studies in survivors of pediatric AKI in patients not treated with ECMO report prevalences of CKD from 6%-59% (15-19). This wide range may be partly explained by the heterogeneity of AKI and CKD definitions, the inclusion of patients with AKI of varying causes, including preexisting renal disease, and the limited sample sizes. Nonetheless, all studies clearly indicate that survivors of AKI during childhood are at risk of residual renal injury.

Several attempts have been made to evaluate renal recovery of survivors of pediatric ECMO. Two studies showed that 96% of survivors of pediatric ECMO who had received RRT recovered renal function before hospital discharge (20, 21). Short-term recovery, however, does not rule out the development of CKD in the long run. To our knowledge, longer-term follow-up of renal injury in children or adults treated with ECMO has not yet been addressed. The aim of our study was, therefore, to determine the prevalence of CKD and evaluate its predictive factors during long-term follow-up of children and adolescents previously treated with neonatal ECMO.

MATERIALS AND METHODS

Setting and participants

This cross-sectional study was conducted in our follow-up clinic between November 2010 and February 2014 and included children who had survived neonatal ECMO (ECMO onset ≤28 days following birth) between January 1, 1992, and December 31, 2012, at our

hospital. We excluded children (1) with inadequate ECMO data, (2) already transferred to adult care, and (3) with congenital abnormalities of the kidneys and urinary tract. When children attended the follow-up clinic more than once, only data of the latest available visit were used.

ECMO was initiated upon reversible severe cardio-respiratory failure and an estimated mortality risk >80% using the criteria reported by Stolar *et al* (22). The ECMO circuit was primed as described elsewhere (23). From 2005 onward, all patients received by protocol pre-emptive continuous hemofiltration (CH) by placement of a hemofilter (Multiflow 60; Hospal, Lyon, France) parallel to the ECMO circuit and distal to the roller pump. Pressure was measured proximal and distal to the filter. The pressure difference was kept constant at 40 mmHg. During CH, the predilution flow rate of the filtration fluid (HF-BIC32; Dirinco, Rosmalen, The Netherlands) was set at the default of 50 mL / kg per hour.

This study was part of a structured prospective post-ECMO follow-up program initiated in 2001, in which lung function, growth and developmental parameters are routinely assessed at ages 1, 2, 5, 8, 12 and 18 years (24, 25). The Medical Ethical Review Board of Erasmus Medical Center provided a waiver for ethics approval. On the basis of Dutch law, the study needs no approval, as it concerns only analysis of data collected in the context of clinical care. Also, no informed consent is needed for such studies (excerpt from the Ethical Review Board's letter: "Medical Research in Human Subjects Act does not apply to this study, since subjects are not being submitted to any handling, nor are there rules of human behaviour being imposed").

Definitions CKD signs and hypertension

CKD was defined as either an abnormal eGFR or proteinuria. During each visit, height, weight, and BP were measured, and blood and urine samples were taken and processed according to standardized protocols for determining serum creatinine (SCr) level (milligrams per deciliter) and urinary protein-to-creatinine (uP / C) ratio (milligrams of protein per milligram of creatinine).

Height was expressed in absolute values and in SD score for chronological age according to growth charts adjusted for country of origin (26). Body mass index (BMI) was defined as weight (kilograms) / height (meters^2) and adjusted for age and gender to give a BMI SDS (27-29). Creatinine was assessed by an enzymatic assay (Creatinine Plus; Roche Diagnostics GmbH, Mannheim, Germany) on a Cobas 8000 analyzer (Roche Diagnostics). Total urine protein concentration was determined by a turbidimetric assay on the Cobas 8000 analyzer.

In all participants, GFR was estimated using the revised Schwartz Equation (0.413×height [centimeters] / SCr) (30). In participants aged >16 years, it was also estimated using the Modification of Diet in Renal Disease (MDRD) formula, because the cohorts used to develop and validate the Schwartz Equation all had very small proportion

of adolescents (30-32). An eGFR <90 mL / min per 1.73m^2 was considered abnormal and staged (CKD stage G1-G5) according to the Kidney Disease Improving Global Outcomes (KDIGO) 2012 Clinical Practice Guideline for the evaluation and management of CKD (33). Glomerular hyperfiltration was defined as an eGFR ≥150 mL / min per 1.73m^2 (34).

Significant proteinuria was quantified as a uP / C ratio >0.50 mg protein / mg creatinine for children age ≤24 months and >0.20 mg protein / mg creatinine for children >24 months (35). If proteinuria was identified, urinalysis was repeated three times in a first morning sample to rule out an orthostatic effect. If all morning samples showed a normal uP / C ratio, midday proteinuria was considered orthostatic, and the participant was scored negative for proteinuria caused by CKD.

Arterial BP measurements (millimeters of Hg) were carried out with a cuff appropriate to the size of the child's upper arm using an electronic device (Dynamap; Critikon, Tampa, FL) three times at 1-minute intervals, with the child seated after 5 minutes of rest. The mean systolic and diastolic BPs of the second and third readings were calculated. Prehypertension and stages 1 and 2 hypertension were defined as a mean systolic and / or diastolic BP between 90th and 95th percentiles, BP ≥95th and ≤99th percentiles or BP >99th percentile, respectively, of reference values for sex, height SDS, and age (36).

Data collection

The following data were collected from medical records: sex, gestational age, birth weight, use of nitric oxide ventilation, vasopressor drugs, and oxygenation index pre-ECMO. In addition, we collected data on the ECMO run and length of ICU stay in our hospital. Ultimately, for each patient, history of AKI was defined according to the maximal SCr-based RIFLE category obtained throughout ECMO: Risk for kidney injury, Injury to the kidney, and Failure of kidney function (3). Because reliable SCr baseline concentrations pre-ICU admission were lacking in most patients, RIFLE categories risk, injury, and failure were defined as SCr >150%, >200%, and >300%, of the median of age-specific SCr reference values, respectively (37). Urinary output criteria were not used to assign AKI severity stages. If CH during ECMO was provided pre-emptively, RIFLE scores could not be reliably estimated because of extrarenal SCr clearance and were scored as missing.

Statistical analysis

Data are expressed as median values with interquartile ranges (IQRs) for continuous variables or numbers with percentages for categorical variables, unless indicated otherwise; 95% binomial confidence intervals (95% CIs) were calculated with the Agresti-Coull method. Univariate overall comparisons between groups using Mann-Whitney U, Pearson chi-squared, or Fisher exact tests, as appropriate, were performed to detect differences between groups (participants versus nonparticipants and CKD versus no CKD) and determine the association between signs of CKD and hypertension. Linear-by-linear

association chi-squared test (or Mantel-Haenszel test for linear association) was used to evaluate the association between history of AKI during ECMO (non-AKI, risk, injury and failure) and CKD signs. Reported are odds ratios with 95% CIs. A two-sided *P* value=0.05 was considered the limit of significance in all analyses. Analyses were performed using SPSS, version 21.

RESULTS

Study population

From 1992 to 2012, 277 of 423 (65%) neonates who received ECMO treatment survived and met eligibility criteria; 53 (19%) children were lost to follow-up for reasons such as non-response or refusal, 50 (18%) children had a scheduled follow-up visit within the study period, and neither SCr nor uP / C was assessed in five (2%) children who did visit the outpatient clinic. These 108 children are referred to as nonparticipants. Ultimately, the study cohort consisted of 169 participants (Figure 1).

Baseline characteristics

Participants versus nonparticipants did not differ in baseline or clinical characteristics (Table 1). The most common diagnoses to initiate ECMO were meconium aspiration syndrome (52%) and congenital diaphragmatic hernia (21%). Median ECMO duration was 119 hours (IQR=84-156 hours). Of all participants, 31 (18%) participants initially received venovenous ECMO. Of 107 participants who received ECMO without pre-emptive CH, evidence of AKI during ECMO was present in 64 patients; 34 (32%) patients were classified as risk, 25 (23%) patients were classified as injury and 5 (5%) patients were classified as failure. Of 62 participants who received ECMO with pre-emptive CH, 12 (19%) participants had SCr levels indicating AKI; however, all were scored missing.

Screening

The median time until screening was 8.2 years (IQR=5.2-12.1 years) after neonatal ECMO. The median SDS height and BMI were -0.40 (IQR=-1.05-0.51) and -0.05 (IQR=-0.75-0.77), respectively.

SCr levels for eGFR were measured in 164 (97%) participants. The median eGFR was 117 mL / min per 1.73m^2 (IQR=104-133 mL / min per 1.73m^2). In nine (5%) children the eGFR was between 90 and 60 mL / min per 1.73m^2, classified as CKD stage G2. None of the participants had an eGFR <60 mL / min per 1.73m^2 Two participants aged >16 years and initially staged as CKD stage G2 using the Schwartz formula had an eGFR>90 mL / min per 1.73m^2 (CKD stage G1) using the MDRD formula. In contrast, 19 (12%) children had an eGFR ≥150 mL / min per 1.73m^2 suggesting hyperfiltration (Figure 2).

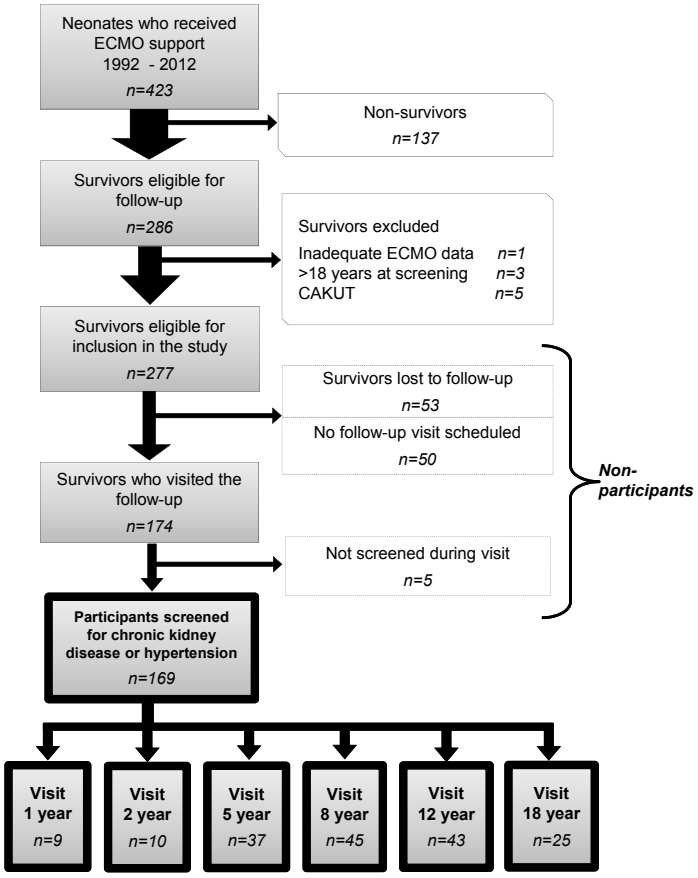

Figure 1. Flowchart of patient recruitment.
Flowchart detailing inclusion and exclusion criteria for children who survived neonatal extracorporeal membrane oxygenation support, which resulted in the final study cohort. CAKUT, congenital abnormalities of the kidneys and urinary tract.

Urine samples were collected in 158 (93%) participants. uP / C ratio was higher in 24 (15%) children, of whom one was <24 months old (uP / C ratio=0.59 mg protein/mg creatinine) and 23 were >24 months old (median=0.26 mg protein/mg creatinine; IQR=0.27-0.39 mg protein/mg creatinine). Also, four participants had orthostatic proteinuria. Hence, 20 (12%) children had persistent proteinuria on repeated testing (median=0.26 mg protein/mg creatinine; IQR 0.23-0.32 mg protein/mg creatinine) (Figure 3).

None of the participants screened had a history of hypertension or were receiving antihypertensive medication. BP was validly measured in 154 (91%) participants. In the remaining 15 participants, measurements were unreliable because of agitation. Sixteen

Table 1. Baseline and clinical characteristics of participants versus non-participants.
RIFLE categories Risk, Injury, and Failure were defined as serum creatinine >150%, >200%, and >300% of the median of age-specific reference values, respectively. RIFLE scores were only assessed among patients treated with neonatal ECMO without pre-emptive continuous hemofiltration. Continuous data are expressed as median (interquartile range), and categorical data are expressed as number (percentage). [a]Assessment of intergroup differences (that is, participants versus nonparticipants) using the Pearson chi-squared or Fisher exact test. [b]Assessment of intergroup differences (that is, participants versus nonparticipants) using the Mann–Whitney test. VV, venovenous; VA, venoarterial; ICU, intensive care unit.

Characteristics	**Participants (*n*=169)**	**Non-participants (*n*=108)**	***P* value**
Sex, female	75 (44)	49 (45)	0.90[b]
Gestational age, weeks	40.0 (38.6 - 41.3)	39.7 (39.0 - 40.9)	0.78[a]
Birth weight, kilograms	3.4 (3.0-3.8)	3.4 (2.9 - 3.8)	0.99[a]
Diagnosis			
Meconium aspiration syndrome	*87 (52)*	*50 (46)*	
Congenital diaphragmatic hernia	*36 (21)*	*13 (12)*	
Isolated persistent pulmonary hypertension	*21 (12)*	*14 (13)*	*0.087*[b]
Sepsis	*9 (5)*	*16 (15)*	
Other	*16 (10)*	*15 (14)*	
Pre-ECMO			
Age at start ECMO (hours)	41 (28 - 73)	42 (28 - 68)	0.84[a]
Vasopressor drugs, yes	161 (95)	102 (94)	0.77[b]
Nitric oxide ventilation, yes	133 (79)	84 (78)	>0.99[b]
Highest oxygenation index	42 (31 - 55)	42 (31 - 56)	0.74[a]
During ECMO			
ECMO mode, VV	27 (16)	10 (9)	0.26[b]
Conversion, VV to VA	4 (2)	3 (3)	
Pre-emptive continuous hemofiltration, yes	62 (37)	32 (30)	0.24[b]
RIFLE scores	**107/169 (63)**	**76/108 (70)**	
Missing RIFLE scores due to pre-emptive continuous hemofiltration	62 (37)	32/108 (30)	
Maximum RIFLE score[b]			
No acute kidney injury	*43/107 (40)*	*32/76 (42)*	
Risk	*34/107 (32)*	*25/76 (33)*	
Injury	*25/107 (23)*	*17/76 (22)*	*0.92*[b]
Failure	*5/107 (5)*	*2/76 (3)*	
Renal replacement therapy	*2/5 (40)*	*1/2 (50)*	
ECMO duration, hours	119 (84 - 156)	129 (86 - 187)	0.16[a]
ICU admission, days	10 (6 - 26)	9 (6 - 17)	0.20[a]

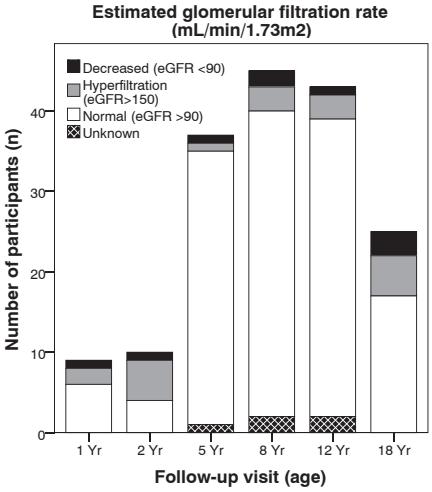

Figure 2. Glomerular filtration rate screening results for all patients stratified by age group. GFR was estimated using the revised Schwartz Equation (0.413×height [centimeters] / serum creatinine). An eGFR <90 mL / min per 1.73m^2 was considered abnormal. Glomerular hyperfiltration was defined as an eGFR ≥150 mL / min per 1.73m^2. GFR, glomerular filtration rate.

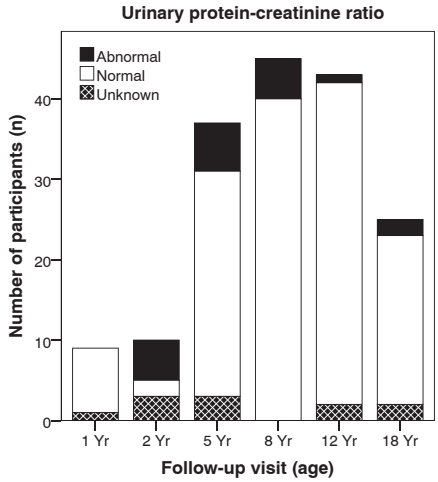

Figure 3. Urinary protein-creatinine screening results for all patients stratified by age group.
Significant proteinuria was quantified as a urinary protein-to-creatinine ratio >0.50 mg protein / mg creatinine for children ages ≤24 months and >0.20 mg protein / mg creatinine for children ages >24 months. If proteinuria was identified, urinalysis was repeated three times in a first morning sample to rule out an orthostatic effect. In case of orthostatic proteinuria, the participant was scored negative for proteinuria. uP / C ratio, urinary protein-creatinine ratio.

(10%) participants met criteria for prehypertension. Twenty-two (14%) participants met criteria of stage 1 hypertension on the basis of either systolic BP (n=16) or diastolic BP (n=6), respectively. Ten (7%) participants met criteria of stage 2 hypertension on the basis of either systolic BP (n=8) or diastolic BP (n=2). Only one participant had hypertension with both systolic and diastolic BP >99th percentile (Figure 4).

Altogether, signs of CKD including either a lower eGFR or higher uP / C ratio, were present in 29 (17%; 95% CI, 12 to 24) participants whereas 32 (19%; 95% CI, 14 to 26) participants had stage 1 or 2 hypertension. Moreover, four participants with a lower eGFR and three participants with proteinuria also presented with hypertension (Figure 5). CKD signs were not associated with hypertension (Fisher exact test, *P*>0.99 for both proteinuria and lower eGFR). None of the children presented with abnormal test results on all three parameters. Also, zero of eight children with BMI >2SDS had hypertension.

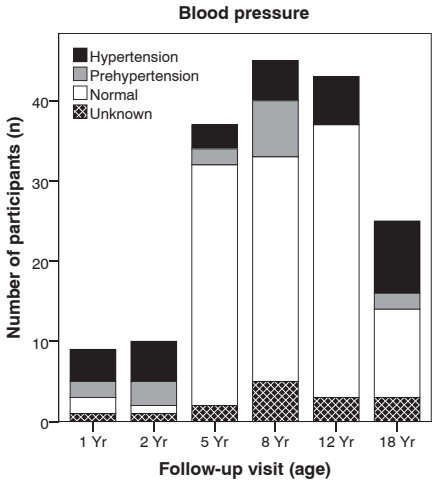

Figure 4. Blood pressure screening results for all patients stratified by age group.
Prehypertension and stage 1 and 2 hypertension were defined as a mean systolic and / or diastolic BP between 90th and 95th percentile, BP ≥95th and ≤99th percentiles, or BP >99th percentiles of reference values for sex, height SD score, and age, respectively. BP, blood pressure.

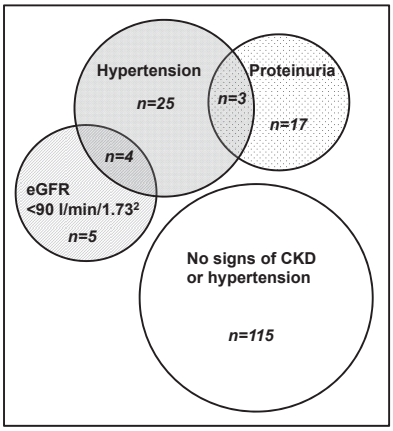

Figure 5. The primary outcome of all participants screened.
GFR was estimated using the revised Schwartz Equation (0.413×height [centimeters] / serum creatinine). An eGFR <90 mL / min per 1.73m^2 was considered abnormal. Significant proteinuria was quantified as a urinary protein-to-creatinine ratio >0.50 mg protein / mg creatinine for children ages ≤24 months and >0.20 mg protein / mg creatinine for children ages >24 months. Prehypertension and stage 1 and 2 hypertension were defined as a mean systolic and / or diastolic BP between 90th and 95th percentile, BP ≥95th and ≤99th percentiles, or BP >99th percentiles of reference values for sex, height SD score, and age, respectively. BP, blood pressure; GFR, glomerular filtration rate; uP / C ratio, urinary protein-creatinine ratio.

Associations CKD

Univariate analysis explored the association between CKD signs or hypertension at follow-up and clinical variables during ICU admission, including diagnosis, nitric oxide ventilation pre-ECMO, ECMO mode and duration, pre-emptive CH during ECMO, and history of AKI during ECMO (Table 2). The clinical variable history of AKI during ECMO was explored solely among 107 participants having received ECMO without pre-emptive CH. A higher proportion of participants with AKI according to the RIFLE criteria had CKD signs or hypertension (22 of 64 participants; 34%) compared to the non-AKI group (nine of 43 participants; 21%; linear-by-linear association chi-squared test, *P*=0.004). Children with RIFLE scores injury and failure had 4.3 times higher odds of CKD signs or hyperten-

sion than those without AKI (95% CI, 1.6 to 12.1; *P*=0.004). None of the other examined variables was associated with CKD (all *P*>0.05) (Table 2).

Table 2. Association between clinical variables and the presence of CKD signs or hypertension (n=169). RIFLE categories Risk, Injury, and Failure were defined as serum creatinine >150%, >200%, and >300% of the median of age-specific reference values, respectively. RIFLE scores were only assessed among patients treated with neonatal ECMO without preemptive continuous hemofiltration. Continuous data are expressed as median (interquartile range, IQR), and categorical data are expressed as number (percentage). [a]Assessment of intergroup differences (that is, non-CKD versus CKD) using the Mann–Whitney test. [b]Assessment of intergroup differences (that is, non-CKD versus CKD) using the Pearson chi-squared or linear-by-linear association chi-squared test. CH, continuous hemofiltration; CKD, chronic kidney disease; VV, venovenous.

Clinical variable	**non-CKD** ***(n=111)***	**CKD or hypertension** ***(n=54)***	***P* value**
Gestational age, *wk*	40.0 (38.6-41.1)	39.7 (39.0-41.0)	0.63[a]
Diagnosis			
Meconium aspiration syndrome	*62 (54)*	*25 (46)*	*0.87*[b]
Congenital diaphragmatic hernia	*22 (19)*	*14 (26)*	
Isolated persistent pulmonary Hypertension	*14 (12)*	*7 (13)*	
Sepsis	*6 (5)*	*3 (6)*	
Other	*11 (10)*	*5 (9)*	
Pre-ECMO			
Age at start ECMO, *h*	41 (26-74)	43 (31-74)	0.50[a]
Vasopressor drugs, *(yes)*	108 (94)	53 (98)	0.23[b]
Nitric oxide ventilation, *(yes)*	92 (80)	41 (76)	0.55[b]
Highest oxygenation index	41 (31-53)	44 (30-58)	0.50[a]
During ECMO			
ECMO mode, VV	20 (17)	11 (20)	0.72[b]
Pre-emptive continuous hemofiltration, yes	39 (34)	23 (43)	0.28[b]
Patients on ECMO without pre-emptive continuous hemofiltration	**(n=76)**	**(n=31)**	
Maximum RIFLE score[b]			
No acute kidney injury	*34 (44)*	*9 (29)*	
Risk	*28 (37)*	*6 (19)*	
Injury	*12 (16)*	*13 (42)*	***0.004***[b]
Failure	*2 (3)*	*3 (10)*	
ECMO duration, *h*	120 (89-159)	105 (74-155)	0.37[a]

DISCUSSION

This is the first study, to our knowledge, prospectively evaluating the prevalence of CKD signs and hypertension during long-term follow-up in a large cohort of children previously treated with neonatal ECMO. A homogeneous study group was created by enrolling only those children who received neonatal ECMO support and excluding children with congenital abnormalities of the kidneys and urinary tract. Given that 54 (32%) participants fulfilled the predefined criteria for CKD or hypertension, this study shows that survivors of ECMO are at increased risk of developing CKD or hypertension, irrespective of AKI history. Still, most values were mildly abnormal, and clinical interventions were not yet needed apart from lifestyle advice or monitoring by a general practitioner. Because CKD progresses very slowly, more advanced CKD may not occur until adulthood. Nevertheless, one must realize that there may be limited renal reserves in these children.

Thirty-two (19%) participants had hypertension, which is considerably higher than a prevalence of 0.3% as was shown in a recent community-based study (38). Because in our study, BP readings were performed during a single visit instead of repetitively, we may have overestimated the hypertension prevalence. Of the clinical variables examined, only history of AKI during ECMO was significantly associated with CKD signs or hypertension. Thus, participants with positive markers of CKD did not differ by diagnosis or other tested clinical variables.

A surprising finding is the high occurrence of hyperfiltration (12%; 19 of 169 participants). This may partially be explained by the relationship between SCr levels and muscle mass. Considering that, in an earlier study from our group, neonatal survivors who had been treated with ECMO showed a significantly reduced exercise capacity, participants may have reduced creatinine generation proportional to a low muscle mass (39). In these patients, creatinine clearance tends to overestimate GFR, whereas the protein-to-creatinine ratio will underestimate protein excretion. Thus, the actual prevalence of CKD may be underestimated in this study.

Prior follow-up studies in critically ill patients not treated with ECMO reported CKD prevalence ranging from 6% to 59% (15-19). The highest incidence (17 of 29 patients; 59%) reported by Askenazi *et al*., in children surviving AKI of varying causes may partly be explained by the inclusion of hyperfiltration in their definition of CKD (15). Mammen *et al.*, in contrast, used a stricter CKD definition (albuminuria and / or eGFR <60 mL / min per 1.73m^2) and found an incidence of 10% after a median follow-up of 1.1 years among 126 patients surviving AKI in the pediatric ICU (17). When applying this strict definition to our cohort, the CKD incidence would have been similar (12%; 19 of 162 participants). Ultimately, Mammen *et al.* reported that one of six (16.6%) patients treated with ECMO surviving AKI had CKD but did not provide detailed clinical data (17).

Major strengths of our study include the large number of participants and the long follow-up time until screening. The follow-up time in previous studies in pediatric ICU survivors was no longer than 3 years, thereby potentially underestimating the true burden of CKD (17). This was not an issue in our study, because 40% of all participants were at least 12 years old. In addition, we used a standard definition to systematically classify AKI during ECMO to explore the potential association with CKD. Children were assigned RIFLE scores during ECMO using SCr values compared to age-appropriate SCr reference values. These SCr reference values were obtained from children without kidney dysfunction daily shortly after birth and at increasing intervals thereafter (37). This method, therefore, allowed us to reliably identify various degrees of AKI during neonatal ECMO support, despite the maternal SCr influence and rapidly changing renal function after birth as well as the absence of baseline SCr concentrations, which was the case with the majority of neonates requiring ECMO.

Several limitations can be identified. First, not all ECMO survivors could be included, because some were lost to follow-up or not scheduled for follow-up within the study period. Nonetheless, baseline characteristics of the participants did not differ from those of the nonparticipants. Hence, we feel that our results can be interpreted without significant selection bias. Second, only a global assessment of chronic kidney damage was performed: GFR was estimated by a formula instead of measured by plasma clearance studies, BP was measured incidentally at the outpatient clinic and not by ambulant BP measurement at home, and no ultrasound study of the kidneys was performed. The methods applied were those that could be relatively easy implemented in our follow-up program. On the basis of these results, future research should include more rigorous methods to gain a more in depth insight on CKD. Third, proteinuria was evaluated according to a uP / C ratio. On one hand, if the denominator (urine creatinine concentration) is low, which might be the case in individuals with a reduced muscle mass, the ratio may easily overestimate proteinuria (40, 41). On the other hand, the prevalence of proteinuria was so much higher than reported in healthy school-aged children and in adolescents who were born preterm that we feel this finding cannot be fully explained by overestimation because of reduced muscle mass (42, 43). Fourth, the association between history of AKI during ECMO and CKD signs or hypertension was solely explored among participants who received neonatal ECMO without CH. The reason is that extra-renal elimination of creatinine precludes the use of SCr levels for AKI assessment during CH, more so because we exclusively used SCr without urine criteria for grading AKI severity (44). Fifth, in contrast to the United Kingdom Collaborative ECMO trial, our study was not a randomized study with controls suffering from severe neonatal cardiorespiratory failure and treated conventionally (45). With only two ECMO centers in The Netherlands, the large majority of neonates with similar severity of illness is treated with

ECMO. Therefore, we could not include a sufficient number of children who survived without ECMO to serve as a control group, because use of historical data is not desirable.

Altogether, this study demonstrates that, after a median follow-up of 8 years, almost one third of all neonatal ECMO survivors have at least one sign of CKD and / or hypertension. To date, the immediate clinical implications are still limited given the only slightly abnormal screening results. Nevertheless, a prevalence of 32%, even higher in children with a prior history of AKI, clearly indicates that these survivors are at risk of developing CKD or hypertension, necessitating adequate treatment given the limited renal reserve. Therefore, even longer-term follow-up, including wide-interval screening for signs of chronic kidney damage in adulthood, is recommended in all patients treated with ECMO.

SUPPLEMENTAL MATERIAL

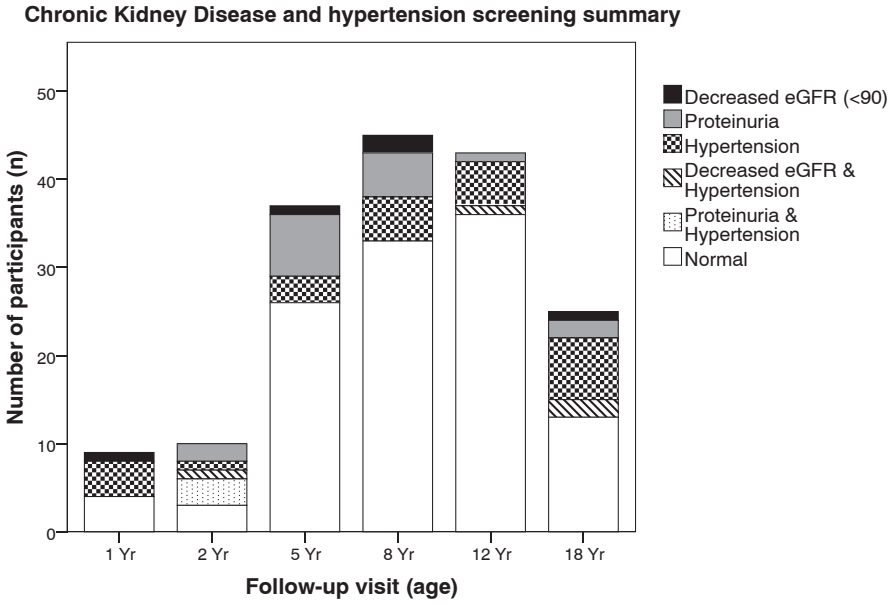

Figure 6. Chronic kidney disease and hypertension screening results per parameter assessed.
GFR was estimated using the revised Schwartz formula (0.413*height [cm]/SCr). An eGFR below 90 mL/min/1.73m^2 was considered abnormal. Glomerular hyperfiltration was defined as an eGFR ≥150 mL/min/1.73m^2.
Proteinuria was quantified as a uP/C ratio >0.50 mg protein/mg creatinine for children aged ≤24 months and >0.20 mg protein/mg creatinine for children older than 24 months. If proteinuria was identified, urinalysis was repeated three times in a first morning sample to rule out an orthostatic effect. In case of orthostatic proteinuria, the participant was scored negative for proteinuria.
Prehypertension, stage 1 and stage 2 hypertension were defined as a mean systolic and/or diastolic blood pressure between 90th and 95th percentile, blood pressure ≥95th and ≤99th percentile or blood pressure >99th percentile, respectively, of reference values for sex, height standard deviation score, and age.
eGFR, estimated glomerular filtration rate; SCr, serum creatinine; uP/C ratio, urinary protein-creatine ratio; mg, milligram; Yr, year.

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PART V

DISCUSSION AND SUMMARY

10 General discussion

CRITICAL ILLNESS

Acute dysfunction of vital organs is a defining aspect of critical illness. The purpose of critical care is to provide life-sustaining organ support (e.g., inotropic therapy for the treatment of hypotension in septic shock) and to rapidly intervene to save organ function (e.g., endotracheal intubation for mechanical ventilation in case of lung injury). For most vital organs, acute injury is tightly correlated with loss of function and, as a result, rapidly diagnosed in clinical practice (e.g., circulatory failure due to cardiogenic shock). Some organs, such as the kidneys, can be severely damaged, however, before any decrease in function becomes clinically apparent. For example, the concentration of serum creatinine (SCr), a functional marker of glomerular filtration rate (GFR) and thus renal function, may remain unchanged until up to 25% – 50% of the functioning nephrons have been lost (1). Hence, early recognition of acute kidney injury is problematic.

ACUTE KIDNEY INJURY

The term 'acute kidney injury' (AKI) has replaced the term 'acute renal failure' and is defined as a rapid deterioration of kidney function, resulting in the inability to maintain fluid, electrolyte and acid-base balance (1). As discussed in the introduction, AKI is a broad clinical syndrome encompassing various etiologies distinguished into three categories: pre-renal (caused by decreased renal perfusion), intrinsic renal (caused by a process within the kidneys e.g., toxic tubular injury), and post-renal (caused by inadequate drainage of urine) (2). Note that these conditions may coexist, especially in patients admitted to an intensive care unit (ICU).

Figure 1 shows an overview of the time course of acute kidney injury in the ICU setting, which is quite similar to the conceptual model described by the Acute Kidney Injury Network (AKIN) (3-5). AKI development in the ICU setting may involve a complex interaction between baseline predisposition, altered intra-glomerular hemodynamics or injury, and tubular damage. Once AKI has developed, supportive therapy and avoidance of secondary renal injury may fail to recover kidney function, which carries the risk of progress to chronic kidney disease (CKD). The key aspects of all models include that only a short time window exists for diagnosing AKI or initiating specific therapies that could reverse AKI. This concept is important as it may aid the development of research questions and consequently our understanding of AKI. There are still wide gaps in our understanding of the epidemiology, the diagnostic value of new serum and urinary biomarkers, and the long-term outcomes of AKI (6, 7). In 2012, Goldstein and colleagues took an important step forward by establishing a Prospective Pediatric AKI Research Group (ppAKI-RG) that focuses on multi-centered research studies dedicated to understanding and treating AKI in pediatric patients.

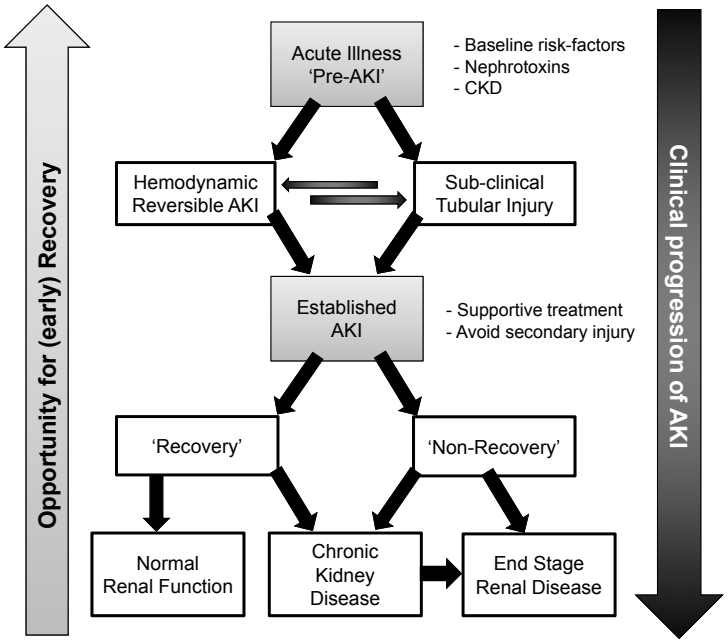

Figure 1. Clinical course of acute kidney injury (adapted from *Prowle et al.* 2014) (3).

Acute kidney injury epidemiology

More than 35 separate quantitative definitions have been used to describe AKI. Still, three features are common to almost all of them: first, an indirect estimate of solute clearance (e.g., SCr doubling or >176 µmol/L); second, urine output over time; and third, exclusion of patients with pre-existing renal dysfunction (8). The lack of a standardized definition has obviously led to a great disparity in the reported incidences of AKI and related mortality, making it difficult to compare study findings (8). It was considered crucial, therefore, to establish an accurate AKI definition that could preferably be used worldwide. In 2004 experts in the field of critical care nephrology developed the RIFLE classification (acronym for Risk, Injury, Failure, Loss of kidney function, and End-stage kidney disease), which was further refined in 2007; the AKIN criteria (9, 10). Both classifications rely on changes in SCr or GFR and/or urine output (UO). Subsequently, Akcan-Arikan and colleagues proposed a RIFLE classification adapted for pediatrics (pRIFLE) (11). The main difference with adult classifications is a lower cut-off SCr value to achieve the F category, which takes into account that children have a lower baseline SCr (7). Nevertheless, since baseline kidney function is often unknown in young children, the studies presented in this thesis classified AKI according to the SCr-based RIFLE score where Risk, Injury, and Failure are defined as SCr concentrations above, respectively, 150%, 200%, and 300% of the median age-specific SCr reference values (12). These SCr

reference values were obtained from children without kidney disease, by using small age intervals ranging from 1 day in the first week after birth up to 3 months at the end of the first year of age.

AKI epidemiology - Critically ill adults

The AKI consensus criteria were then largely validated in terms of determining the incidence of AKI and its prognostic stratification in several settings. Hoste and colleagues were one of the first to provide an overview of the epidemiology of AKI in hospitalized adults (6). In the 13 reviewed publications more than 100,000 patients were assessed for AKI by means of the RIFLE criteria. The rate of AKI varied from 10.8% to 100%, dependent on the type of cohort studied (Table 1). In patients admitted to the ICU, AKI occurred in two thirds of all admissions and 4% to 5% of ICU patients were treated with renal replacement therapy (RRT) for AKI (6). As a rough comparison, in their study the population incidence of AKI among hospitalized patients paralleled the incidence of severe sepsis whereas the incidence of patients treated with RRT for AKI paralleled the incidence of acute respiratory distress syndrome (6).

An extensive review of the association between AKI and mortality was published by Ricci and colleagues (13). They analysed 13 studies evaluating over 71,000 hospitalized adults and found a stepwise increase in the risk ratio (RR) for death, progressing from non-AKI to Risk (RR=2.40) to Injury (RR=4.15) to Failure (RR=6.37, $P<0.0001$ for all) (13). Other studies also showed an association between AKI and adverse outcomes both on the short-term, such as longer ICU stay and mortality, and on the long-term, such as chronic kidney disease or cardiovascular disease (14-17).

Table 1. Overview of epidemiological AKI studies in adult ICU patients.
AKI, acute kidney injury; AKIN, Acute Kidney Injury Network; ICU, intensive care unit.

First Author	Cohort	Adult Patients, n	AKI, %	Risk AKIN I, %	Injury AKIN II, %	Failure AKIN III, %
Cruz (107)	ICU	2,164	10.8	2.1	3.8	4.9
Heringlake (108)	Cardiac surgery-ICU	29,623	16	9	5	2
Uchino (109)	Hospital	20.126	18	9.1	5.2	3.7
De Geus (110)	ICU	663	27.3	11.6	6.6	9.0
Ostermann (111)	ICU	41,972	35.8	17.2	11	7.6
Lopes (112)	Sepsis	182	37.4	6.0	11.5	19.8
Ahlstrom (113)	ICU	685	52.0	25.5	15.2	11.2
Hoste (114)	ICU	5,383	67	12.4	26.5	28.1

AKI epidemiology - Critically ill children

Since the validation of AKI definitions in adults, populations have been studied as well using AKI definitions based on either pRIFLE or AKIN criteria. In critically ill children, reported incidences also appear to vary widely from 4.5% to 82.0% dependent on the definition used and the population studied (11, 18-21) (Table 2). Similar to studies in adults, AKI was found associated with poor outcomes, such as prolonged mechanical ventilation, increased ICU length of stay, and pediatric ICU mortality (18, 20, 22).

Chapter 6 describes a large prospective cohort study evaluating the AKI incidence in children younger than one year, term-born, admitted to the ICU and requiring mechanical ventilation. More than half of all patients (51%) developed AKI at any time during ICU admission, most of them within 2 days of ICU entrance. Ackan-Arikan and colleagues reported a considerably higher AKI incidence of 82%. This higher incidence may be explained by the role of compromised systemic hemodynamics leading to AKI, since all patients in their study received vasopressor drugs whereas this was the case for only half of our cohort (4). The opposite holds true for the studies of Bailey and colleagues and Alkandari and colleagues, who reported a significantly lower AKI incidence of 4.5% and 17.9%, respectively (18, 19). More specifically, these groups conducted a retrospective analysis of all pediatric ICU admissions over a certain period, including non-ventilated children, whereas we exclusively enrolled children who required endotracheal intubation and mechanical ventilation for respiratory failure. In addition, we used a less strict AKI definition than Bailey and colleagues, who defined AKI as doubling of baseline SCr.

Notably, of the 35 patients in our study who developed AKI within 48 hours following admission, almost 70% already had AKI on admission. These figures are in line with a large retrospective cohort study evaluating 2106 pediatric ICU admissions (18). Both studies also show that AKI predominantly occurs in the first three days, and notably in the very first day of ICU care (18).

Table 2. Overview of epidemiological AKI studies in pediatric ICU patients.
AKI, acute kidney injury; AKIN, acute kidney injury network; ICU, intensive care unit.

First Author	Cohort	Pediatric Patients, n	AKI, %	Risk AKIN I, %	Injury AKIN II, %	Failure AKIN III, %
Bailey (13)	ICU	1.047	4.5	-	-	-
Schneider (20)	ICU	3.396	10	3.3	3	3.7
Alkandari (18)	ICU	2.106	17.9	9.8	4.3	3.8
Zwiers (chapter 6)	ICU	101	51	28.7	11.9	10.9
Plotz (21)	ICU	103	58	30.1	21.4	6.8
Zapitelli (58)	ICU	150	75.7	33.3	20.1	16.7
Akcan-Arikan (11)	ICU	150	82.0	40.0	21.3	20.7

AKI epidemiology - Extracorporeal membrane oxygenation

Patients in need of extracorporeal membrane oxygenation (ECMO) suffer from severe respiratory or cardiovascular failure not responding to conventional intensive care and, as such, are probably most at risk for AKI. Chapters 4, 5 and 7 report AKI incidences among patients treated with ECMO, with or without pre-emptive continuous hemofiltration (CH). In a historical cohort of 242 neonates receiving ECMO *without* pre-emptive CH, 64% had evidence of AKI, with 11% qualifying as Failure (chapter 4). The last RIFLE score before ECMO decannulation or on ECMO day 12 indicated that only 46% of all AKI patients had improved at least one RIFLE category. These results differ from those of other studies focusing on ECMO-treated patients (Table 3) (23-25). One very large study evaluated approximately 10,000 ECMO patients registered in the ELSO Registry and reported a much lower AKI incidence rate varying from 8% in neonatal patients to 21% in pediatric patients (24). One of the reasons explaining this discrepancy is that the ELSO Registry traditionally collects cross-sectional information, with the possibility that the AKI episode has been missed. Besides, the ELSO Registry defines AKI as a SCr level >1.5 mg/dL. Though in newborns this cut-off value may include lower grades of AKI, in infants older than 2 days this definition corresponds only to severe cases of the RIFLE category Failure, resulting in an underestimation of AKI. A study by Smith and colleagues demonstrated a slightly higher incidence of 72% of AKI in 48 pediatric cardiac patients requiring ECMO (25). In that study children required ECMO for circulatory support whereas a substantial proportion of our patients required ECMO for ventilatory support in spite of adequate cardiac function (42% had meconium aspiration syndrome). Another study, in 68 neonates with congenital diaphragmatic hernia (CDH) requiring ECMO, reported an

Table 3. Overview of epidemiological AKI studies in patients treated with extracorporeal membrane oxygenation .
AKI, acute kidney injury; AKIN, Acute Kidney Injury Network; hrs, hours.

First Author	Cohort	Patients, n	AKI, %	Risk AKIN I, %	Injury AKIN II, %	Failure AKIN III, %
Askenazi (24)	ECMO-neonates	10,116	8.0	-	-	-
Askenazi (24)	ECMO-pediatric	2778	20.5	-	-	-
Zwiers (Chapter 7)	ECMO-children	31	58.1	19.4	12.9	25.8
Zwiers (115)	ECMO-children	242	63.2	29.8	22.7	10.7
Lin (28)	ECMO-adults Cardiac Surgery	46	78.3	15.2	39.1	23.9
Kielstein (116)	ECMO-adults	200	60	-	-	60
Gadepalli (23)	ECMO-neonates CDH	68	71	-	22	49
Smith (25)	ECMO-children Cardiac Surgery	48	71.7	-	-	-

AKI incidence of 71% which is nearly equal to an incidence of 69% as found in our CDH patients (chapter 4) (23).

In children who received ECMO *with* pre-emptive CH, we found that 58% (18 out of 31 patients) had any rise in SCr levels indicating AKI at some point prior to, during or after ECMO treatment (chapter 7). This somewhat lower percentage may be explained by the insertion of CH in the ECMO circuit, which provides extrarenal SCr elimination, leading to an underestimation of the AKI incidence and its severity.

Interestingly, we identified two clinical factors associated with AKI, i.e., not receiving inhaled nitric oxide (NO) ventilation pre-ECMO and younger age at the start of ECMO (chapter 4). The former suggests that NO ventilation pre-ECMO might protect against AKI. Inhaled NO modulates the distribution of the systemic blood flow and thereby potentially of renal perfusion. However, hypotheses on how changes in renal perfusion are related to the development of AKI are contradictory (26).With respect to the latter, younger age at the start of ECMO, there are a number of aspects of neonatal renal physiology that are pertinent to AKI in younger neonates, including the stage of nephrogenesis and thus number of functioning nephrons, renal blood flow, GFR, and immaturity of tubules to respond to homeostatic need (27). Still, since the age difference was not more than a few days, the concept of basic renal physiology differences is considered irrelevant. Finally, survival until ICU-discharge was significantly lower for patients in the Failure category than for the other patients. This high mortality risk confirms the previously reported association between AKI and mortality (20, 23-25, 28-30), and supports the concept that patients may benefit from early recognition of AKI and prevention of deterioration of renal function.

CONCLUSIONS

AKI is a frequent and serious complication in critically ill children, and is associated with poor outcomes, such as prolonged mechanical ventilation, increased length of ICU stay, and mortality.

BIOMARKERS

Even though consensus criteria for AKI categorization are now widely accepted, one must realize that a proportional SCr increase still forms the basis of all criteria. This is problematic since, as argued in the introduction, SCr is generally considered a delayed and unreliable indicator of AKI for several reasons. SCr is subject to confounding factors, such as age and muscle mass (9). Moreover, with a decline of renal function the propor-

tion of creatinine excretion due to active tubular secretion increases (10-12). SCr-based definitions of AKI have limited relevance for neonates, whose SCr level reflects the maternal creatinine level at birth and normally drops over the first weeks of life dependent on gestational age (10). Furthermore, in ECMO-treated patients, SCr levels will be diluted on account of the large blood volume in the extracorporeal circuit. When ECMO-treated patients receive CH –either on indication or pre-emptively– extrarenal SCr elimination further masks early stages of renal injury. These important shortcomings of SCr have driven the search for novel early, more sensitive and specific biomarkers of AKI.

Reference values of alternative biomarkers

Correct interpretation of the concentrations of new AKI biomarkers in critically ill children requires the availability of reliable reference values. Chapters 2 and 3 present reference values for children up to one year of age, for GFR marker beta-trace protein (BTP) as well as the tubular injury biomarkers neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1).

We have shown that BTP concentrations peak at birth and then decline until the age of one year (chapter 2). The physiological course of BTP as presented in our study is in accordance with previous findings and strongly suggests an effect of renal maturation. Levels established in our study are slightly lower than those of Bariciak and colleagues, who studied term-born neonates measured at a younger postnatal age (31). The age-related decline in BTP levels established in our study fits the pattern reported by Bökenkamp in children aged 2 to 21 years (32). Similar to Bokenkamp and co-workers, we found relatively wide BTP reference ranges (all subjects, 5^{th} – 95^{th} centile 0.27 – 1.38), which may limit interpretation of abnormal values in daily clinical practice. The trend toward lower levels with increasing age was confirmed by a study in which adults showed the lowest BTP levels (33).

The correlation between BTP and SCr levels in our infant cohort was weak but significant. One explanation for the weak correlation might be that BTP in principle does not cross the placental barrier, while SCr does (34). As a result BTP concentrations at birth exclusively reflect the neonate's GFR, whereas SCr levels mainly reflect maternal kidney function. In this light, BTP might offer a promising and easy-to-use alternative to SCR-based methods for estimating GFR in newborns.

In chapter 3, uNGAL and KIM-1 levels for children up to one year of age are presented. uNGAL levels found in our study are in line with data reported by Cangemi and colleagues for a similar study cohort, but substantially higher than reported for older children by Bennett and colleagues (35, 36). uNGAL showed a clear developmental change. It peaked after birth, then gradually decreased to a nadir of 13.8 and 26.0 ng/mL in boys and girls, respectively, at 6 months, and remained low towards the end of the first year of life. These high levels early in life are surprising because uNGAL is normally expressed

at very low levels in healthy renal tissue but up regulated in response to tubular injury growth (37-42). One explanation for the age-related change in uNGAL levels could be the normal physiological renal maturation, as NGAL has been shown to act as a growth and differentiation factor in multiple cell types, including developing mature renal epithelia in rats (43). The precise molecular mechanism through which NGAL exerts its growth effects on renal cells is not yet known however (44).The gender difference in this study is another important finding: uNGAL levels in girls were higher than in boys (chapter 3). This was also reported for young adults, children and very low body weight neonates (24, 36, 45-47). Huyn and colleagues suggested that urine from bagged and voided specimens from girls might be contaminated with vaginal secretion containing neutrophils (45).

Chapter 3 also demonstrates that KIM-1 concentrations are extremely low or undetectable (below 0.08 ng/ml) in almost all healthy children ≤1 year (median KIM-1 0.06 ng/mg urinary creatinine). Hence, KIM-1 may be a clinically useful marker for early proximal tubular damage as any rise in urine concentration indicates injury. Unfortunately, all previous studies in healthy subjects used different type of KIM-1 assays, which makes comparison across studies difficult (46-50). In contrast to uNGAL, there was no gender difference pertaining to KIM-1 levels in our cohort, which confirms findings on this issue by Bennett and colleagues (36).

There is an on-going debate on the normalization of urinary AKI biomarkers concentrations to urine creatinine (uCr) concentration to account for creatinine clearance and urine flow. The pitfalls of reporting absolute concentrations of a urinary biomarker are easy to identify: oliguria will cause an increase, and polyuria a decrease in biomarker concentration. Normalizing a urinary biomarker concentration to uCr takes into account differences in urinary flow rate. However, when normalizing, one must also take into account the other determinant of uCr, the rate of creatinine excretion by the kidney. In the setting of AKI, which is considered a non-steady-state condition, the uCr excretion rate changes over time, and may overestimate AKI (51, 52). We therefore decided to present absolute biomarker concentrations rather than uCr normalizations for our group of critically ill children (chapter 6). For children treated with ECMO and pre-emptive CH, on the other hand, we decided to normalize urinary AKI biomarker concentrations to uCr (chapter 7). Fluid overload is common in ECMO patients and associated with worse clinical outcomes (53) (54). Since most patients are fluid-overloaded when ECMO is initiated, forced diuresis is pursued once the patient is stable on ECMO (54, 55). Normalization of urinary AKI biomarker concentrations to uCr is essential to account for variations in water retention throughout ECMO.

Critically ill children

As described in chapter 6, uNGAL and KIM-1 levels in critically ill children increase following ICU admission and peak at 6 to 12 hours and 12 to 24 hours, respectively. These patterns resemble those reported in adult studies (56). De Geus and colleagues published a well-conducted cohort study of adult ICU patients (n=543) and indeed demonstrated that upregulated uNGAL and KIM-1 protein concentrations increased over time post-admission (56). Similar to our study, uNGAL in their study differentiated between non-AKI and AKI patients right from the time of admission. KIM-1 levels differentiated between non-AKI and AKI at the 24 hour time point for the first time whereas in our study this was also the case within 6 hours post-admission (56). A severity of illness bias due to case selection might explain the dissimilarities in biomarker performances, seeing that De Geus and colleagues excluded patients with sepsis, whereas this was the primary diagnosis for 14% of our patients (56).

To the best of our knowledge there are no pediatric studies available on the patterns of biomarkers using small time-intervals (6 hours) following ICU admission. One study in 13 asphyxiated newborns reported uNGAL and KIM-1 levels on days 1 and 3 of life (57). Notably, uNGAL levels in this study were increased on day 1 and remained stable thereafter whereas KIM-1 levels were high on day 1 as well, but substantially decreased at 24 to 36 hours until 48 to 72 hours, the last measurement (57).

In Chapter 6 we also showed that, while KIM-1 levels remained steady, uNGAL in eleven patients, who developed AKI at a later time-point, peaked at 6 to 12 hours before reaching the diagnosis of AKI. A similar pattern for uNGAL levels was reported by De Geus and colleagues for adults (56). Likewise, Zappitelli and colleagues reported that uNGAL levels in 21 pediatric ICU patients relative to the day of pRIFLE AKI attainment peaked one day before AKI onset (58). These findings are important as they suggest that uNGAL is a better early marker for AKI than SCr, and thus allows for a more timely start or adjustment of renoprotective therapy

We also studied urinary NGAL and KIM-1 in 31 children who received ECMO support combined with pre-emptive CH (chapter 7). Median uNGAL and KIM-1 concentrations during ECMO greatly exceeded age- and gender specific reference values – indicating substantial tubular damage – although they were lower than those reported in previous studies (59, 60). The different biomarker assays used may partially explain the differences.

In our study neither uNGAL nor KIM-1 levels differed between survivors and non-survivors. This is conflicting with the study of Wai and colleagues who found lower median uNGAL/uCr concentrations in survivors than in non-survivors (60). Wai and colleagues mainly enrolled children with sepsis, which is known to rapidly induce massive upregulation of NGAL expression in both serum and urine (37,38). Eighty per cent of our non-survivors, however, suffered from major life-threatening congenital anomalies

(e.g., hypoplastic left heart syndrome or congenital diaphragmatic hernia) rather than sepsis. NGAL levels in our non-survivors may therefore not adequately predict risk of death.

Krawczeski and colleagues were the first to suggest that biomarker combinations improved prediction of AKI beyond clinical models based on clinical factors, e.g., age and time on CPB (61). Their data show that uNGAL levels were significantly increased at 2 hours while KIM-1 significantly increased only at 12 hours post- cardiopulmonary bypass (CPB). This temporal relationship of uNGAL and KIM-1 is quite similar to that found in our study following ECMO cannulation, with KIM-1 increasing also 12 hour later than uNGAL post-cannulation, and peaking approximately 24 hours later than uNGAL.

CONCLUSIONS

Current consensus criteria for diagnosing AKI, based on changes in SCr and UO, lack sensitivity and specificity, which has driven the search for new biomarkers. The urinary biomarkers NGAL and KIM-1 are significantly increased in children requiring mechanical ventilation following ICU admission. In children who later will develop AKI, uNGAL level peaks 6 to 12 hours before the first recorded rise in SCr level indicating AKI. In ECMO-treated children, the temporal pattern of both biomarkers supports the finding of previous studies that biomarker combinations may be useful for identifying different stages of AKI.

Limitations of biomarkers used

Conceptually, the biomarker development process can be fragmented into three stages: discovery or exploratory, 'probable' valid, and 'known' valid stage. In the first stage, analytical techniques are used to identify potential candidate biomarkers in a given patient population, typically with clear clinical phenotypes, by a single center approach. Once a candidate biomarker is identified, biomarker validation should be initiated, which is a lengthy and complex process, to ensure that the biomarker is 'reliable for intended use'. It starts with internal validation (within the initial cohort), followed by external validation (a second independent cohort) to reach the 'probable' valid stage. The biomarker may reach the 'known' valid stage after initial phase 1 and 2 clinical trials, followed by larger prospective clinical trials (62, 63).

Both AKI biomarkers, uNGAL and KIM-1, have successfully passed through the preclinical assay development and initial clinical testing phases ('probable' valid stage) of the biomarker development process, to which our studies have also contributed. They have now entered the prospective screening stage, facilitated by the development of

commercial tools for the measurement of these biomarkers in large populations across different laboratories. Still, several important limitations can be recognized with respect to the use of uNGAL and KIM-1 (41, 64, 65).

First, although the majority of NGAL in urine derives from the injured kidney, non-renal NGAL sources might adversely affect the diagnostic accuracy (37). In fact, NGAL concentrations may be influenced by co-existing conditions such as hypertension, systemic infections, inflammations, hypoxia and malignancies, and urinary tract infections, which may limit its diagnostic performance across several clinical settings (58, 66-69). However, the levels of urine NGAL in these situations are significantly lower than those typically measured in AKI (41). The same holds true for KIM-1, as previous studies reported that KIM-1 expression was up regulated, too, in other diseases than AKI, such as diabetic nephropathy, renal cancer as well as immune disorders (64, 70).

Apart from these methodological limitations, there are technical concerns related to the use of NGAL and KIM-1 as AKI biomarkers. The method of biomarker measurement is expensive and is not yet readily available 24/7, which hampers its routine use in the clinical setting. Still, one can imagine that costs of measuring biomarkers now in the 'probable' valid stage may be justified once they enter the 'known' valid stage and eventually may allow for earlier effective treatment in terms of fluid retention and overload. By then, a traditional cost-effectiveness analysis should be performed to compare the relative costs and outcomes of biomarker-guided therapies.

Obtaining fresh clean-catch urine samples, particularly in children who have not been toilet-trained, is difficult and not always successful. Other methods used to collect urine, including gauzes placed in the diaper may lead to inaccurate results due to contamination with faeces and vaginal secretion containing neutrophils (45, 71). In critical care the majority of these concerns are addressed, for example by the use of indwelling bladder catheters. Even then, however, obtaining a urine sample of sufficient volume can be problematic in case of hypotensive shock or severe AKI with oliguria or anuria. The inability to obtain sufficient volume of urine has contributed mostly to missing samples in our studies.

With regard to storage, NGAL and KIM-1 are sufficiently stable in urine if stored at 4°C for a maximum of 48 hours, subsequently frozen for several months at -80°C, but not at -20°C (72, 73). Although this limitation is not relevant for routine clinical practice, it should be considered in future research.

Current status of NGAL and KIM-1

The current status of NGAL was recently evaluated by Haase and colleagues by reviewing 58 studies including more than 16,500 patients in three common clinical settings: cardiac surgery, critical illness and kidney transplantation (74, 75). Irrespective of the clinical setting, both urine and plasma NGAL were predictive of AKI and its severity, with

an overall AUC of 0.79–0.87. More importantly, NGAL significantly improved the prediction of AKI risk over the clinical model alone in all three clinical settings investigated. The authors noted some limitations, including lack of published studies that adhere to diagnostic study guidelines (STARD guidelines), heterogeneity in AKI definition, the lack of uniformly applicable cut-off values, and variability in the performance of commercially available NGAL assays (75).

The diagnostic value of KIM-1 was evaluated by Shao and colleagues in a meta-analysis. A total of 2979 patients from 11 studies were enrolled in the analysis. For all studies, measurement of KIM-1 appeared a relative good discriminator for AKI with an estimated AUC of 0.86 (0.83-0.89). Still, the small number of studies, heterogeneity in study populations and variable AKI definitions include important limitations. Also, different biomarker assays and cutoff values were used. Thus, the potential diagnostic and prognostic value of KIM-1 need to be validated further in large cohort studies and clinical settings, especially in the setting of critical care (65).

CONCLUSIONS

Urinary AKI biomarkers NGAL and KIM-1 have successfully passed the 'probable' valid stage of biomarker development in both adults and children, to which our studies have also contributed, and entered the prospective screening stage. Still, their clinical application is associated with important limitations related to sample collection, processing and storage as well as inter-individual and intra-individual patient variability due to coexisting diseases.

Renoprotective treatment of AKI

Even though novel biomarkers of AKI are moving closer towards clinical relevance, evidence is still lacking on renoprotective regimens. Different approaches are applied, including pharmacological and fluid management interventions.

Pharmacological interventions

Removal or minimization of potential nephrotoxic drugs (e.g., non-steroidal anti-inflammatory drugs, aminoglycosides) is the most important first step to avoid or limit impeding kidney failure. Once AKI develops, drug treatment remains primarily supportive by dose adjustment of (partially) renally eliminated drugs (e.g., midazolam) in view of the fact that there is no specific drug therapy proven to prevent development progression of AKI at this time.

Several drugs, e.g., clonidine, aspirin, and fenoldopam, have been studied in large randomized placebo- controlled trials in adult patients undergoing cardiac surgery. The rationale for the use of these three drugs (vasodilator, antiplatelet agent, anti-inflammatory drug, respectively) was based on targeting the presumed mediators of renal injury in the postoperative period: renal vasoconstriction with medullary ischemia, microthrombosis, and inflammation. Despite earlier positive results in both animal models and small studies, these large trials failed to show benefit and instead indicated harm (76). Increased rates of dialysis (fenoldopam), mortality at 30 days (fenoldopam), hypotension (clonidine, fenoldopam) and gastrointestinal bleeding (aspirin) were found in patients randomized to treatment (76). Noteworthy, AKI was significantly more common among patients experiencing these adverse effects. This relevant AKI mechanism is not being targeted and questions our current understanding of the mechanisms driving AKI. Explanations for the lack of benefit could be the timing of drug administration (it occasionally served as secondary prevention), the intensity of the inflammatory cascade among the study population, or the inherent adverse effects of the specific drugs administered (76).

An alternative therapy is the infusion of low-dose ("renal dose") dopamine to improve suboptimal renal perfusion. Even though small clinical trials suggested a benefit from low-dose dopamine, several large randomized clinical trials showed that its use is ineffective for AKI in both adult and pediatric patients (77-80). In contrast, it was even demonstrated that low-dose dopamine can reduce renal blood flow in critically ill adults with AKI and thus may have the opposite effect (81).

Fluid management

Critically ill patients' hemodynamic status requires careful attention because hypotension and hypovolemia may contribute to or worsen kidney injury. Increasing evidence suggests that for critically patients isotonic crystalloids should be used instead of colloids for initial expansion of intravascular volume (82). The timing and amount of fluid dosing to prevent AKI and other organ damage is still debated. There is a delicate balance between maintaining an optimal fluid balance without excessive interstitial fluid overload while still ensuring stable circulatory parameters. In this context, Goldstein and colleagues proposed a fluid accumulation 3-phase conceptual model for the patient with, or at risk for AKI (83). The model fragments fluid management strategies into 3 phases based on the clinical status: (Phase 1) fluid resuscitation/repletion; (Phase 2) maintenance of fluid balance and/or prevention of fluid overload; and (Phase 3) fluid removal. In general the goals of fluid therapy are defined for each phase and include for example central venous pressure and cardiac output goals. ICU staff are regularly faced with difficult fluid-related clinical decisions regarding (1) the transition from Phase 1 (resuscitation) to Phase 2 (maintenance); and (2) the dilemma that occurs when a patient

with fluid overload develops AKI (83). If an AKI patient cannot tolerate the needed fluid volumes without developing fluid overload, choices have to be made between fluid restriction, diuretic administration and RRT initiation. This is essential because a positive fluid balance after hemodynamic stabilization is thought to contribute to oxygenation failure and mortality (84). Since fluid restriction may decrease optimal caloric intake, however, which could be detrimental to overall outcome, in the third phase diuretics may provide a non-invasive way to achieve a targeted fluid balance. Thus, to increase UO and reduce fluid overload diuretics are frequently administered but have never been shown to prevent AKI or improve outcomes of patients who developed AKI (85, 86). In fact, in critically ill adults, a model adjusted for several covariates such as age, gender, severity of illness, indicated that the use of diuretics during AKI was associated with higher risk of death and non-recovery of renal function (87).

Fluid management is even more challenging in ECMO-treated patients, who are not only at high risk for developing AKI but in most cases also already fluid overloaded prior to ECMO cannulation (chapter 4) (53). Reduction of fluid overload by means of concomitant CH in these patients was found to improve lung function and shorten the time to decannulation but the effect on renal outcome was not determined (88, 89). This is why we evaluated 80 children treated with ECMO and pre-emptive CH to assess the prevalence and risk factors of AKI following ECMO decannulation (chapter 5). All children received CH pre-emptively and diuretics were prescribed at the attending physician's discretion if a targeted fluid balance or UO of >0.5 ml/kg/hour could not be achieved. Despite the fact that CH was in place for fluid management, 81% of all patients received diuretics of whom 77% more than once. In addition, the furosemide equivalent (furosemide + bumetanide*40) dose administered was the only covariate associated with AKI following ECMO (chapter 5). This confirms previous findings in children undergoing cardiac surgery with CPB, which is to some extent similar to ECMO. Chiravuri and colleagues showed that children with poor renal outcomes were 4-12 times more likely to have received diuretics (90). Our data raise questions about the clinical benefit of administering loop diuretics to patients receiving ECMO and pre-emptive CH. Obviously, diuretics were prescribed in our study cohort for oliguria, which may have been due to AKI or due to intravascular volume depletion as a result of fluid removal by CH. Excessive fluid removal however, either by diuretics, CVVH or combination therapy, may increase the risk of pre-renal AKI, potentially followed by acute tubular necrosis (53).

CONCLUSIONS

Several renoprotective strategies have been evaluated but none has been proven to be effective to prevent or treat evolving AKI. Thus, in a critical ill child with AKI, management includes supportive care consisting of treating the cause where possible, monitoring fluid and electrolyte balance closely, and optimizing hemodynamic status with appropriate fluid therapy. Special attention must be paid to the use of loop diuretics during ECMO. Especially since AKI in the context of hypovolaemia may be aggravated by excessive fluid removal by additional diuretics or by concomitant CH.

PROGRESSION TO CHRONIC KIDNEY DISEASE

Severe AKI can result in a substantial loss of functioning nephrons, which may lead to hyperfiltration in the spared ones (91). This compensatory response will initially maintain the glomerular filtration rate (GFR) but may progress to interstitial fibrosis and tubulus atrophy. These pathologic changes may eventually lead to chronic kidney disease (CKD), a condition characterized by a progressive decline in GFR over time, proteinuria, and/or systemic hypertension (92). Timely therapeutic interventions can slow the progression of renal injury and medication should be titrated to actual clearance (93, 94).

To date, research has largely focused on short-term implications of AKI in the ICU. The few follow-up studies in survivors of pediatric AKI who did not receive ECMO support report prevalence's of CKD from 6% – 59% (95-99). This wide range may be partly explained by the heterogeneity of AKI and CKD definitions, the inclusion of patients with AKI of varying causes including pre-existing renal disease, and limited sample sizes. Nonetheless, all studies clearly indicate that survivors of AKI during childhood are at risk of residual renal injury (95-99).

In chapter 9 we demonstrated that one third of the children previously treated with neonatal ECMO fulfilled the predefined criteria for CKD or hypertension. Therefore this study demonstrates that ECMO survivors are at increased risk of developing CKD or hypertension. Still, most values were mildly abnormal and clinical interventions were not yet needed, apart from lifestyle advice and blood pressure monitoring by a general practitioner. Also, the follow-up was already maximally 18 years. Since CKD progresses very slowly, more advanced CKD may not occur until adulthood (100, 101). One must realize that there may be limited renal reserve in these children. Therefore, even longer-term follow-up including wide-interval screening for signs of chronic kidney damage into adulthood is recommended in all patients treated with ECMO.

CONCLUSIONS

Data on long-term consequences of pediatric AKI are limited to date. The findings of this thesis however clearly indicate that ECMO-treated children, especially those with a prior history of AKI, are at risk of developing CKD or hypertension, necessitating adequate treatment given the limited renal reserve.

FUTURE PERSPECTIVES

An ideal AKI biomarker should be non-invasive, easy-to-use, able to utilize standardized clinical platforms, consistent in repetitive measurement with a high sensitivity and specificity for early detection, and having a wide dynamic range and cut-off values to allow for risk stratification (102). Considering these criteria, urinary NGAL and KIM-1 seem to be both suitable but have strengths and weaknesses and remain to be validated in large clinical studies. One must realize that children in particular provide a valuable and informative cohort for AKI studies since they do not have many of the common complicating co-morbidities highly prevalent in adults (atherosclerotic heart disease, smoking, diabetes mellitus, chronic obstructive pulmonary disease, etc.)

To summarize, what we learned from previous studies is that optimal timing for serial measurements in the proper clinical context (i.e. right patient, right clinical setting) is crucial. Yet, optimal timing is particularly difficult in critically ill patients, for whom the exact onset of AKI is largely unknown. Besides, the etiology of AKI in the ICU setting is often multifactorial (e.g., ischemic insults, nephrotoxic drugs and sepsis). The importance of "appropriately timed biomarker measurements" is further underlined by the surprising observation that almost two thirds of the critically ill AKI patients in our studies already suffered from AKI – indicated by SCr-based definitions – on ICU entrance. Hence, future studies focusing on an early biomarker-guided diagnosis of AKI in the ICU setting should perform measurements within the first hours of hospital admission. If possible following emergency department presentation or on admission to the general pediatric ward but at least before ICU entrance. In this light, rapid NGAL testing by means of point-of-care testing will be instrumental in improving ongoing patient management as well as triage decision-making (103). The primary advantage of point-of-care testing is its ability to provide rapid test results and, thus, facilitate earlier treatment and avoidance of well-known risk factors for AKI (e.g., aminoglycosides, neuromuscular blocking agents). With regard to children, however, another important advantage includes the smaller volumes of specimen needed for testing. An even more promising concept has been

developed for KIM-1: a urine dipstick (the RenaStick), which is generally considered a desirable screening tool in clinical practice, especially in young children (104). Although this dipstick requires further clinical validation, important advantages of this technique over currently available laboratory methods can be recognized including the greater ease of use, visual readout, and rapid detection.

Seeing that repair and recovery of kidney structure following AKI is a very complex process, biomarkers that could serve as indicators of repair would be valuable for clinical decision-making throughout the disease process. We recently conducted a pilot study in 20 critically ill children to evaluate epidermal growth factor (EGF), a urinary renal recovery biomarker (chapter 8). Notably, in our study EGF levels on ICU admission did not differ from EGF levels following the onset of renal recovery (decrease in SCr concentration of ≥0.3 mg/dL). One explanation might be the degree of recovery. In fact, we evaluated renal recovery at 7 days following admission, which might have been too short for EGF to increase. This explanation is further emphasized by the findings of Hoffman and colleagues. They studied neonates receiving therapeutic hypothermia and found significantly increased EGF/uCr concentrations in the samples collected at >24 hours post-recovery (59).

The fact that AKI etiology and timing are often unclear suggests that more than one novel biomarker may be necessary to obtain sufficient sensitivity and specificity to predict AKI (prior to changes in SCr) but also to adequately monitor renal recovery. Thus, future studies should focus on a panel of multiple biomarkers consisting of NGAL for the early detection of AKI, and EGF for monitoring of renal recovery.

If AKI can be anticipated or detected early by biomarkers, preventive measures could be evaluated. Still, there are no proven therapies to prevent or treat AKI in any clinical context. Consequently, physicians working in the ICU are limited to supportive measures for the critically ill patient who develops AKI, such as vasoactive medication provision and fluid administration to support blood pressure and organ perfusion, and in severe cases of AKI even RRT to restore fluid homeostasis. In general, AKI is not considered by many pediatric ICU specialists at admission of individual patients. As a consequence, early fluid restriction such as decreasing total IV fluids, is usually considered "too late" in the course of the disease. This has significant consequences for the need for respiratory support and the length of stay in the ICU.

One way to further evaluate renoprotective strategies is to measure biomarker concentrations as surrogate endpoints instead of SCr, which is still considered a delayed and insensitive measure of AKI. Given the high sensitivity of biomarkers for AKI such as NGAL, especially in a homogenous group of patients, a biomarker may be particularly useful in preventive strategies. Ricci and colleagues took the lead and performed a randomized trial of intraoperative fenoldopam to prevent AKI, using NGAL and Cystatin C to diagnose AKI in pediatric cardiac surgery patients (105). Although they did evaluate a drug

that was not demonstrated beneficial as secondary prevention in adults with early-stage AKI, they are the first to select postoperative urine NGAL and cystatin C concentrations as primary end-points (105, 106). Obviously, the sample size was small; still this study provides a model to use AKI biomarkers in a novel manner, which could also be applied in the critical care setting.

CONCLUSIONS

Although KIM-1 remains to be validated in large clinical studies across several clinical settings, for NGAL a multimodal approach of clinical and NGAL-assisted decision-making for AKI prediction and treatment is likely to be a most promising next step.

This is of utmost importance as early detection of AKI by means of biomarker concentrations as surrogate endpoints, instead of SCr, may be a promising step towards evaluation of renoprotective strategies. Until then, physicians should focus on providing optimal supportive, renoprotective care to critically ill patients with or at risk for AKI. Since the dynamic nature of critical illness will require careful reassessment of the risks and benefits of potential pharmaceutical interventions and fluid management strategies, physicians would do well to constantly remind themselves "*Do not squeeze the kidneys!*"

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11 Summary
Samenvatting

SUMMARY

The aims of this thesis are to establish reference values of a new set of biomarkers of kidney function and tubular injury in infants without kidney injury; to evaluate acute kidney injury (AKI) in critically ill infants, grouped by treatment with or without extracorporeal membrane oxygenation (ECMO), using RIFLE-criteria as well as urinary biomarkers; and to determine the prevalence and predictive factors of chronic kidney disease (CKD) during long-term follow-up of children previously treated with neonatal ECMO.

PART I. Introduction

Chapter 1 describes the categorisation of AKI in pre-renal, renal and post-renal causes. It is argued that an early AKI diagnosis is essential to enable the most appropriate management strategies, which should be initiated before irreversible renal damage occurs. Literature is reviewed on the traditional gold standard for measuring glomerular filtration rate (GFR) and the new concepts in AKI definition, which include consensus criteria based on serum creatinine (SCr) and urine output e.g. "RIFLE criteria" (Risk, Injury, Failure, Loss- and End-stage renal disease). Since especially the use of SCr has several important limitations, there has been considerable interest recently in identifying new urinary AKI biomarkers, of which the most extensively studied ones are introduced in this chapter.

PART II. Reference values

In **Chapters 2 and 3** reference values are presented of the glomerular filtration biomarker serum BTP, and the tubular injury biomarkers urinary neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1). No reference values have been reported for children ≤1 year of age, age-stratified according to shorts intervals including intervals as short as months. The study cohort consisted of basically healthy full-term children (at least 37 weeks of corrected gestational age at the time of entry in the study) who were randomly selected from those admitted to the general pediatric or pediatric surgical ward. Prior to the study start, health status was determined for each subject on the basis of medical history and physical examination by the attending physician to exclude illnesses including bacterial or viral infections. The findings are important for interpretation of the biomarker studies described in Part III.

PART III. Acute kidney injury – Etiology & Biomarkers

Children with cardio-respiratory failure requiring ECMO were the subjects in the studies presented in **Chapters 4, 5,** and **7**. ECMO temporarily provides cardiopulmonary support that allows time for evaluation, diagnosis, and treatment of the condition causing the cardio-respiratory failure. **Chapter 4** shows that two thirds of neonates treated with ECMO suffered from AKI and that AKI severity was associated with mortality. The clinical

course of AKI during ECMO is even more concerning as only 46% of all patients initially classified as AKI showed some degree of renal recovery during ECMO. These results strongly support the idea that patients may benefit from early recognition of AKI and prevention of deterioration of renal function. The study described in **Chapter 5** focusses on AKI following ECMO in children who also received pre-emptive continuous hemofiltration (CH) as standard of care. The prevalence of AKI post-ECMO was remarkably high, but renal function recovered in most survivors at ICU discharge or transfer. Despite the fact that CH was in place for fluid management, diuretics were administered to no less than 81% of all ECMO-treated children. Although this study could not draw conclusions about cause-and-effect relationships, the independent association between the use of diuretics and AKI post-ECMO warrants special attention to the administration of diuretics during ECMO with pre-emptive CH.

Recent studies on new urinary biomarkers including NGAL and KIM-1 as possible alternatives to SCr for the early detection of AKI inspired the two large prospective studies described in **Chapters 6 and 7**. **Chapter 6** showed that urinary NGAL and KIM-1 levels in critically ill children requiring mechanical ventilation increase following ICU admission, and peak 6 to 12 hours and 12 to 24 hours, respectively, thereafter. It also appeared that almost 70% of the children who developed AKI within 48 hours following admission, had already AKI when entering the ICU. Still, urinary NGAL was reasonably able to discriminate between infants who developed AKI versus those who did not. **Chapter 7** further supports the use of a biomarker panel rather than a single biomarker to identify patients at risk of AKI, especially those receiving ECMO combined with continuous hemofiltration as in these patients the shortcomings of traditional markers of kidney damage are even more prominent. **Chapter 8** evaluated urinary biomarkers associated with renal recovery in a subgroup of the cohort prospectively studied in **Chapter 6**, i.e. a subgroup of critically ill children. It was shown that urinary NGAL and fibroblast growth factor 2 (FGF-2) significantly decreased within 4 to 7 days following ICU admission. Renal recovery biomarker epidermal growth factor (EGF), however, did not change over this time period.

PART IV. Progression to chronic kidney disease

Since AKI during childhood may predispose for CKD in adulthood, we evaluated long-term follow-up (median 8 years) of kidney function in neonatal ECMO survivors (**Chapter 9)**. It became clear that neonatal ECMO survivors, especially those who had AKI, are at risk of developing CKD or hypertension necessitating adequate treatment, given the limited renal reserve.

PART V. General discussion

Chapter 10 contains the general discussion. The contents of this thesis are reviewed in connection with the literature. It is concluded that **A)** AKI is a frequent and serious complication in critically ill children, especially those treated with ECMO; **B)** current consensus criteria for diagnosing AKI lack sensitivity, which has driven the search for new biomarkers including urinary NGAL and KIM-1; **C)** uNGAL and KIM-1 are significantly increased in children requiring mechanical ventilation following ICU admission when compared to age- and gender specific reference values; **D)** among those critically ill children who developed AKI, NGAL levels peaked 6 to 12 hours before any rise in SCr levels had been recorded for the first time; and **E)** data on long-term consequences of pediatric AKI are scarce. The findings of this thesis add to the knowledge base, however, as they clearly indicate that ECMO-treated children, especially those with a prior history of AKI, are at risk of developing CKD or hypertension.

Since the most promising AKI biomarkers have both strengths and weaknesses, the question of how to optimally use them in clinical routine is still open. Biomarkers trials in which the clinical context is fully incorporated are expected to provide clues to their ideal utilization (i.e. right patient, right clinical setting). Hence, for an early AKI diagnosis in the critical care setting, samples should be collected within the first hours of hospital admission, if possible even before entering the ICU. Furthermore, the heterogeneity of AKI in children admitted to the ICU suggests that a multiple biomarker approach is required, rather than a single biomarker, to capture different phases of AKI and guide therapeutic strategies. Ultimately, measuring biomarker concentrations, as surrogate endpoints in addition to SCr, may allow for the evaluation of renoprotective strategies for prevention of AKI development and/or AKI progression in an earlier stage.

SAMENVATTING

Dit proefschrift beschrijft verschillende studies gericht op het voorkomen en beloop van acute nierschade (AKI) bij ernstig zieke kinderen die opgenomen zijn op de intensive care (IC), al dan niet behandeld met extracorporale membraan oxygenatie (ECMO). Enerzijds wordt AKI geëvalueerd met behulp van het serum creatinine en de RIFLE-criteria, anderzijds met behulp van nieuwe biomarkers voor de nierfunctie en voor schade aan de tubuli. Tot slot wordt beschreven wat de prevalentie en voorspellende factoren zijn van chronische nierziekte (CKD) bij kinderen die op neonatale leeftijd ECMO behandeling hebben ondergaan.

DEEL I. Inleiding

Hoofdstuk 1 beschrijft de indeling van AKI naar pre-renale, renale- en post-renale oorzaken. Er wordt beargumenteerd dat de diagnose AKI in een vroeg stadium essentieel is om de beste behandeling toe te kunnen passen zodat het optreden van onomkeerbare nierschade wordt gereduceerd dan wel voorkomen. Er wordt een literatuuroverzicht gepresenteerd over de traditionele gouden standaard voor het meten van de glomerulaire filtratiesnelheid (GFR) en over nieuwe opvattingen wat betreft de definitie van AKI. Deze opvattingen betreffen consensuscriteria – gebaseerd onder andere op de concentratie van creatinine in serum (SCr) en de urineproductie – zoals de "RIFLE criteria" (acroniem voor Risk, Injury, Failure, Loss- and End-stage renal disease). Omdat het gebruik van SCr belangrijke beperkingen heeft, is er toenemende aandacht voor het vinden van nieuwe biomarkers. De biomarkers die vandaag de dag het meest uitgebreid zijn onderzocht, zullen geïntroduceerd worden in dit hoofdstuk.

DEEL II. Referentiewaarden

In de **hoofdstukken 2 en 3** worden referentiewaarden gepresenteerd voor serum β-trace protein (BTP) als biomarker voor de glomerulaire filtratiesnelheid, en neutrophil gelatinase-associated lipocalin (NGAL) en kidney injury molecule-1 (KIM-1) als biomarkers voor tubulaire schade. Tot op heden zijn er nog geen referentiewaarden gepubliceerd voor kinderen jonger dan 1 jaar oud, gestratificeerd naar leeftijd in maanden. Deze studie betrof een cohort van relatief gezonde a-terme kinderen (gecorrigeerde zwangerschapsduur ten minste 37 weken) die willekeurig werden geselecteerd uit kinderen opgenomen op de algemene pediatrische of pediatrische chirurgische afdeling. De gezondheidsstatus van de geselecteerde kinderen werd voorafgaand aan de studie bepaald aan de hand van een anamnese en lichamelijk onderzoek. Dit werd verricht door de dienstdoende kinderarts om op deze manier bijvoorbeeld de aanwezigheid van bacteriële of virale infecties uit te sluiten. De gevonden referentiewaarden zijn belangrijk voor de interpretatie van de biomarker studies die in deel III worden beschreven.

DEEL III. Acute Nierschade - Etiologie & Biomarkers

Kinderen met cardiorespiratoir falen waarvoor ECMO noodzakelijk was, waren onderwerp van de studie zoals beschreven in de **hoofdstukken 4, 5 en 7**. ECMO – ofwel kunstlongbehandeling – biedt tijdelijke ondersteuning van het hart en de longen, hetgeen de gelegenheid biedt voor evaluatie, diagnose en behandeling van de aandoening die het cardiorespiratoir falen veroorzaakt. **Hoofdstuk 4** laat zien dat twee derde van de pasgeborenen behandeld met ECMO lijdt aan AKI en dat de ernst daarvan geassocieerd is met mortaliteit. Het klinische verloop van AKI tijdens ECMO is zorgwekkend: bij slechts 46% van alle patiënten die bij opname al aan AKI leden werd tijdens ECMO enige mate van herstel van de nierfunctie gezien. Deze resultaten ondersteunen de gedachte dat vroegtijdige herkenning van AKI verslechtering van de nierfunctie kan voorkomen. Het in **hoofdstuk 5** beschreven onderzoek richt zich op AKI bij kinderen die naast ECMO ook met continue hemofiltratie werden behandeld. De prevalentie van AKI gemeten na deze behandeling was opvallend hoog, alhoewel de nierfunctie in de meeste gevallen was hersteld bij IC ontslag. Ondanks dat continue hemofiltratie werd gebruikt voor management van de vochtbalans, kreeg maar liefst 81% van de kinderen diuretica toegediend. Hoewel er in dit onderzoek geen conclusies getrokken kunnen worden over de oorzaak-gevolgrelatie, geeft de onafhankelijke associatie tussen het gebruik van diuretica en het optreden van AKI na ECMO-behandeling aan dat de toediening van diuretica tijdens ECMO gecombineerd met continue hemofiltratie speciale aandacht behoeft.

Recente studies over nieuwe biomarkers in de urine – waaronder NGAL en KIM-1 – als mogelijke alternatieven voor SCr voor de vroege opsporing van AKI, waren de aanleiding voor de twee grote prospectieve studies beschreven in de **hoofdstukken 6 en 7**. Bij ernstig zieke, beademde, kinderen namen de NGAL en KIM-1 concentraties in de urine toe na opname op de IC en piekten na respectievelijk 6 tot 12 uur en 12 tot 24 uur **(Hoofdstuk 6)**. Ook bleek dat bijna 70% van de kinderen die binnen 48 uur AKI ontwikkelden, al AKI had ten tijde van de opname. Desondanks kon uit de NGAL-concentratie in de kinderen die bij opname nog geen AKI hadden, worden opgemaakt welke kinderen later AKI zouden ontwikkelen en welke niet. **Hoofdstuk 7** laat zien dat het gebruik van een combinatie van biomarkers – in plaats van één enkele biomarker – mogelijk effectiever is bij patiënten met een verhoogd risico op AKI, vooral degenen die met ECMO en continue hemofiltratie worden behandeld, aangezien bij deze patiënten de tekortkomingen van traditionele markers nog meer op de voorgrond treden. **Hoofdstuk 8** evalueert een andere groep biomarkers, mogelijk geassocieerd met minder goed herstel van de nierfunctie, in een subgroep van het cohort ernstig zieke kinderen zoals bestudeerd in **hoofdstuk 6**. Er werd aangetoond dat de concentraties van zowel NGAL als fibroblast growth factor-2 (FGF-2) significant dalen binnen 4 tot 7 dagen na IC-opname. De concentratie van epidermale growth factor (EGF), eveneens een mogelijke biomarker voor herstel van nierfunctie, bleef echter ongewijzigd gedurende deze periode.

DEEL IV. Progressie naar chronische nierziekte

Aangezien het doormaken van AKI op de kinderleeftijd kan predisponeren voor het ontwikkelen van CKD jaren later, hebben we gedurende de poliklinische follow-up de nierfunctie geëvalueerd van kinderen die op neonatale leeftijd met ECMO waren behandeld (mediane follow-up periode 8 jaar na ECMO behandeling) (**hoofdstuk 9**). Het werd duidelijk dat deze kinderen, vooral degenen die AKI hebben doorgemaakt, een verhoogd risico hebben op het ontwikkelen van CKD of hypertensie waarvoor adequate behandeling noodzakelijk is. Aangezien deze schade op de langere termijn kan toenemen, is het langdurig vervolgen van deze kinderen geïndiceerd.

DEEL V. Algemene discussie

Hoofdstuk 10 bevat de algemene discussie. De inhoud van dit proefschrift wordt beoordeeld in de context van bestaande literatuur. Geconcludeerd wordt dat: A) AKI een veelvoorkomende ernstige complicatie is bij kritisch zieke kinderen, vooral degenen die behandeld worden met ECMO; B) de huidige consensuscriteria voor de diagnose van AKI een beperkte gevoeligheid hebben, wat heeft geresulteerd in een zoektocht naar nieuwe biomarkers; C) de concentraties van NGAL en KIM-1 in de urine van kritiek zieke beademde kinderen na IC-opname aanzienlijk hoger zijn dan referentiewaarden voor leeftijd en geslacht; D) onder de ernstig zieke kinderen die AKI ontwikkelden, piekte de NGAL concentraties 6 tot 12 uur voordat de SCr-concentratie steeg en E) er een verhoogd risico is op CKD jaren na ernstige ziekte gedurende de neonatale periode, hetgeen wordt versterkt door het doorgemaakt hebben van AKI.

Aangezien de meest veelbelovende AKI-biomarkers zowel sterke als zwakke punten hebben, blijft de vraag hoe ze optimaal gebruikt kunnen worden in de klinische context onbeantwoord. Studies waarin de klinische context volledig is geëvalueerd zullen naar verwachting meer duidelijkheid geven daaromtrent (d.w.z. juiste patiënt, juiste klinische setting). Om het mogelijk te maken AKI in een vroeg stadium te diagnosticeren dienen urinemonsters verzameld te worden binnen de eerste uren van ziekenhuisopname, zo mogelijk vóór opname op de IC. Bovendien lijkt gezien de heterogeniteit van de aandoening een combinatie van biomarkers mogelijk beter dan één enkele biomarker, omdat daarmee de verschillende stadia van AKI kunnen worden vastgesteld en de behandeling daaraan kan worden aangepast. Uiteindelijk biedt het meten van biomarker concentraties als surrogaateindpunt naast SCr de mogelijkheid om therapieën ter voorkoming van nierschade en/of nierschade-progressie in een eerder stadium te evalueren.

PART VI

APPENDICES

List of abbreviations

About the author

List of publications

PhD Portfolio

Dankwoord

LIST OF ABBREVIATIONS

AIC	Akaike information criterion
AKI	Acute kidney injury
AUC	Area under the curve
BP	Blood pressure
BTP	β-trace protein
CAKUT	Congenital anomalies of the kidney and urinary tract
CDH	Congenital diaphragmatic hernia
CH	Continuous hemofiltration
CI	Confidence interval
CKD	Chronic kidney disease
CPB	Cardiopulmonary bypass
CV	Coefficient of variation
CysC	Cystatin C
ECMO	Extracorporeal membrane oxygenation
EGF	Epidermal growth factor
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
ELSO Registry	Extracorporeal Life Support Organization Registry
FO	Fluid overload
FGF-2	Fibroblast growth factor 2
GAMLSS	Generalized Additive Model for Location, Scale and Shape
GFR	Glomerular filtration rate
HF	Hemofiltration
HIVAN	HIV-associated nephropathy
ICU	Intensive care unit
IQR	Inter quartile range
KDIGO	Kidney Disease Improving Global Outcomes
Kg	Kilogram
KIM-1	Kidney injury molecule-1
L	Liter
LOD	Limit of detection
LOS	Length of stay
LoQ	Limit of quantification
MAS	Meconium aspiration syndrome
mg	Milligram
mL	Milliliter
n	Number

ng	Nanogram
NGAL	Neutrophil gelatinase-associated lipocalin
NO	Nitric oxide
N.R.	Not reported
NS	Nonsurvivors
OR	Odds ratio
PDMS	Patient Data Management System
PIM	Pediatric Index of Mortality
PPHN	Persistent pulmonary hypertension
PRISM	Pediatric Risk of Mortality Score
RIFLE	Risk injury failure loss end-stage renal disease
ROC curve	Receiver operating characteristics curve
RRT	Renal replacement therapy
RSV	Respiratory syncytial virus
S	Survivors
SCr	Serum creatinine
SD	Standard deviation
SDS	Standard deviation score
SE	Standard error
SPSS	Statistical Package for the Social Sciences
uCr	Urinary creatinine
uKIM-1	Urinary kidney injury molecule-1
uNGAL	Urinary neutrophil gelatinase associated lipocalin
uP / C	Urinary protein-creatinine
VA	Venoarterial
VV	Venovenous

ABOUT THE AUTHOR

Alexandra Johanna Maria Zwiers was born in Amsterdam, The Netherlands, on November the 10th 1985. She received her Athenaeum degree at Veenlanden College in Mijdrecht in 2004. Before starting her medical training, she attended one year of Biomedical Sciences at Utrecht University and obtained a propaedeutic degree. In 2005 she started her medical training at the Erasmus MC - University Medical Center in Rotterdam. Alexandra combined her study with several extra-curricular activities including a board membership of the Student introduction committee of Utrecht University. In her third year of medical school she arranged an Elective public health internship and went to Tanzania, Africa, to educate community groups in local villages and raise awareness about HIV, tuberculosis, and malaria.

She finished the theoretical part of medical school, "doctoraal examen", in October 2009 (master's degree). Her graduate research focused on pain and sedation in children treated with extracorporeal membrane oxygenation. Under supervision of Prof. Tibboel, Dr. K. Cransberg, and Dr. S.N. de Wildt, she prepared a grant application for the Sophia Foundation for Scientific Research, which was fortunately honored. She then commenced her PhD project on the short- and long-term consequences of acute kidney injury in critically ill infants requiring intensive care. From 2011 she was a board member of PLAN (national Platform AIOs/Post-docs Nephrology), part of the Dutch Federation of Nephrology, where she represented Erasmus University and organized symposia, workshops and presentations. In the third year of her PhD research Alexandra was awarded a Kolff-grant by the Dutch Kidney Foundation, which enabled her to conduct a research project at Children's Research Institute, Children's National Medical Center, Washington DC, USA under the guidance of Prof. J.N. van den Anker and Prof. P.E. Ray.

In 2014 Alexandra started her internships at the Erasmus MC - University Medical Center in Rotterdam.

LIST OF PUBLICATIONS

1. Buijs EA, **Zwiers AJ**, Ista E, Tibboel D, de Wildt SN. *Biomarkers and clinical tools in critically ill children: are we heading toward tailored drug therapy?* Biomark Med. 2012 Jun;6(3):239-57.

2. **Zwiers AJ**, de Wildt SN, Hop WC, Dorresteijn EM, Gischler SJ, Tibboel D, Cransberg K. *Acute kidney injury is a frequent complication in critically ill neonates receiving extracorporeal membrane oxygenation: a 14-year cohort study.* Crit Care. 2013 Jul 24;17(4):R151.

3. **Zwiers AJ**, Cransberg K, van Rosmalen J, Wildschut ED, Tibboel D, de Wildt SN. *Loop diuretics are an independent risk factor for acute kidney injury in children on extracorporeal membrane oxygenation with pre-emptive continuous hemofiltration.* Intensive Care Med. 2014 Apr;40(4):627-8.

4. Buijs EA, Reiss IK, Kraemer U, Andrinopoulou ER, **Zwiers AJ**, Ince C, Tibboel D. *Increasing mean arterial blood pressure and heart rate with catecholaminergic drugs does not improve the microcirculation in children with congenital diaphragmatic hernia: a prospective cohort study.* Pediatr Crit Care Med. 2014 May;15(4):343-54.

5. **Zwiers AJ**, Cransberg K, de Rijke YB, Willemsen SP, de Mol AC, Tibboel D, de Wildt SN. *Reference ranges for serum β-trace protein in neonates and children younger than 1 year of age.* Clin Chem Lab Med. 2014 Dec;52(12):1815-21.

6. **Zwiers AJ**, IJsselstijn H, van Rosmalen J, Gischler SJ, de Wildt SN, Tibboel D, Cransberg K. *CKD and hypertension during long-term follow-up in children and adolescents previously treated with Extracorporeal Membrane Oxygenation.* Clin J Am Soc Nephrol. 2014 Dec 5;9(12):2070-8.

7. **Zwiers AJ**, de Wildt SN, de Rijke YB, Willemsen SP, Abdullahi NS, Tibboel D, Cransberg K. *Reference intervals for renal injury biomarkers neutrophil gelatinase-associated lipocalin and kidney injury molecule-1 in young infants.* **Accepted for publication** - Clin Chem Lab Med 2015, February.

8. **Zwiers AJ**, Cransberg K, van Rosmalen J, de Rijke YB, Tibboel D, de Wildt SN. *Urinary NGAL is associated with renal injury following Extracorporeal membrane oxygenation and concomitant CH.* **Accepted for publication** - Pediatr Crit Care Med 2015, March.

9. **Zwiers AJ**, de Wildt SN, van Rosmalen J, de Rijke YB, Buijs EA, Tibboel D, Cransberg K. *Urinary neutrophil gelatinase-associated lipocalin identifies critically ill young children with acute kidney injury following intensive care admission: a prospective cohort study.* Crit Care. 2015 Apr 21;19(1):181.

10. Buijs EA, Ince C, **Zwiers AJ**, Andrinopoulou ER, Mooij MG, Verboom EM, Houmes RM, Wildschut ED, Reiss IKM, Tibboel D. *The microcirculation in children with primary respiratory disease requiring venoarterial or venovenous extracorporeal membrane oxygenation: a prospective cohort study.* **Submitted for publication.**

PHD PORTFOLIO

Name PhD student:	A.J.M. Zwiers
Erasmus MC department:	Intensive Care and Department of Pediatric Surgery
PhD period:	2010 – 2015
Promotor:	Prof. dr. D. Tibboel
Copromotors:	Dr. K. Cransberg Dr. S.N. de Wildt

	Year	Workload (ECTS)
General academic skills		
BROK (Basiscursus Regelgeving Klinisch Onderzoek) Erasmus University Rotterdam	2010	1.0
CPO mini-course	2010	0.3
Systematic Literature Search and Endnote	2010	0.6
Molmed – Research Management for PhD students	2011	1.0
Biomedical English writing and communication	2011	4.0
Integrity in Scientific Research	2012	1.5
Molmed – Writing successful Grant Applications	2013	0.5
Molmed – Research Management for Post-docs	2014	1.0
Research skills		
Nihes - Introduction to clinical research	2010	0.9
Nihes - Biostatistics for clinicians	2010	1.0
MolMed – Basic Introduction Course on SPSS	2010	1.0
Principles of Clinical Pharmacology (NIH web conference course)	2010 - 2011	2.0
Molmed – R Statistical Package	2011	1.4
MolMed - Biomedical Research Techniques Xth edition	2011	1.5
Winterschool 'One week Nephrology', Dutch Federation of Nephrology	2012	1.4
Nihes - Repeated measurements in Clinical Studies	2012	1.4
Pediatric Clinical Pharmacology Interactive Webinar - Sumner J. Yaffe Memorial Lecture Series (NIH web conference course)	2012 - 2013	1.0
Molmed – Survival analysis for MD's	2013	0.5
Symposia and workshops		
Star Child Health Summit, AMC, Amsterdam, The Netherlands Workshop: *Bias in Pediatric Trials & Developmental Pharmacological Aspects*	2010	0.3
1st Erasmus Critical Care Days	2011	0.3
22th annual meeting of the European Society of Pediatric and Neonatal Intensive care (ESPNIC), Hannover, Germany Workshop: *Simulation in the Intensive Care Unit*	2011	0.3
Laboratory Research Techniques, Washington DC, USA	2012	1.0
Young Investigator Day (TULIPS/NVK)	2013	0.3
Presenting Skills for junior researchers 2nd series	2013	1.0

	Year	Workload (ECTS)
National conferences		
Spring meeting Nederlandse Sectie Intensive Care Kinderen: *oral presentation*	2012	0.3
Sophia research day: *oral presentation & poster presentation*	2013	1.0
2nd Erasmus Critical Care Days: *invited speaker*	2013	1.0
Najaars-symposium Dutch Federation of Nephrology, Utrecht: *short oral poster presentation* (first prize winner)	2013	0.6
PLAN symposium "The Rotterdam experience": organization and oral presentation	2013	1.0
International conferences		
6th Pediatric continuous renal replacement therapy congress (PCRRT), Rome, Italy: *oral presentation* (young oral presenter award)	2010	1.5
22th Annual meeting of the ESPNIC, Hannover, Germany: *oral presentation (2x)*	2011	1.5
7th PCRRT, Cincinnati, USA: *poster presentation (2x)*	2012	1.5
24th annual meeting of the ESPNIC, Rotterdam, The Netherlands: *oral presentation & poster presentation*	2013	1.5
16th Congress of the International Pediatric Nephrology Association (IPNA), Shanghai, China: *short oral presentation & poster presentation*	2013	1.5
Euro-ELSO 2014 & International ECMO Course, Paris, France: *invited speaker*	2014	1.5
Euro-ELSO 2015 – 4th International Congress, Regensburg, Germany: *poster presentation* (young fellow award)	2015	1.0
Teaching activities		
Supervising medical student master's thesis (*n=3*)	2011 - 2012	1.6
Teaching medical students (2nd year)	2013	0.6
Other		
Board of the 'Sophia Onderzoekers Vertegenwoordiging (SOV)'	2010 - 2012	3.0
Organizer Pediatric Pharmacology Research Meeting	2011 - 2012	0.3
Pharmacology days, Erasmus MC-Sophia, Intensive Care and Department of Pediatric Surgery (annually): *oral presentations (5x)*	2010 - 2013	1.5
Writing F1000 evaluations (*n=8*)	2013	1.2
Writing several grant proposals	2010 - 2014	1.0
Pediatric Pharmacology Research Meetings: *multiple oral presentations*	2010 - 2014	2.0
Board (representing Erasmus University) of PLAN (*Platform AIOs/Post-docs Nephrology)*, Dutch Federation of Nephrology	2011-2014	4.0
Pediatric Nephrology Research Meetings	2012 - 2014	1.5

ECTS = European Credit Transfer and Accumulation System
1 ECTS represents 28 hours

PROLOOG

In feite is promoveren net als snowboarden: adembenemende besneeuwde bergtoppen, gevolgd door een gevarieerde afdaling naar een soms wat laag gelegen dal. Het is het uitzicht vanuit de gondel –de professor– die je doet realiseren dat er daadwerkelijk overzicht is, en nog belangrijker: dat de energie die je er in steekt het allemaal meer dan waard is.

De eerste keer dat je op een snowboard staat –de eerste keer dat je een wetenschappelijk artikel leest, laat staan wilt schrijven– gaat gepaard met vallen en opstaan. Het lijkt in eerste instantie een onmogelijke opgave om je er succesvol doorheen te slaan, maar al snel blijken er ervaren mensen te zijn die je kunnen helpen om in balans te blijven –je copromotoren– om vervolgens de eerste meters enthousiast af te leggen. En ja hoor, je bent verkocht!

Het begint met een blauwe piste –een onderzoeksprotocol– en je raakt vertrouwd met de sneeuw. De afdaling gaat steeds soepeler –en behendig weet je na diverse amendementen de METC te passeren. Op de piste wordt er met regelmaat genoten van een Café met een Crêpe –oftewel Doppio, Starbucks en Koekela– waarna ook de rode pistes en gletsjers worden getrotseerd.

Tijdens de volgende dagen in de boardercross draait het om het overleven van ijzige sprongen, gladde schansen en andere ogenschijnlijke onmogelijke hindernissen –te vergelijken met de daadwerkelijke uitvoering van het onderzoek; het includeren van patiënten, met onmisbare hulp van verpleegkundigen samples verzamelen en opslaan, waarna de analyses, interpretatie van resultaten, het voeren van vele discussies en schrijven volgen.

Snowboarden met een blauwe, zonnige, lucht terwijl er zojuist een flink pak verse poeder sneeuw is gevallen geeft het euforische gevoel waar je het allemaal voor doet –te vergelijken met het moment waarop je hoort dat een artikel is geaccepteerd. Als je het hele skigebied –je promotie traject– hebt verkend eindig je op de zwarte piste: een steile, niet geprepareerde afdaling die de adrenaline in je lichaam maximaal doet stijgen: je trotseert de verdediging van je proefschrift. Dit is het moment waarop je eindelijk mag gaan bewijzen dat je ook hier gecontroleerd maar met veel plezier kan afdalen! Tot slot dien je de dag af te sluiten met een gezellige après-ski…

DANKWOORD

Deze reis heb ik niet alleen gemaakt en daarom wil ik dan ook velen bedanken. Allereerst, de patiënten en ouders die bereid waren in een zeer moeilijke periode in te stemmen met deelname aan onderzoek. De vele verpleegkundigen die gedurende nacht en ontij zowel bloed als urine op nauwgezette tijden hebben verzameld. Uiteraard de stafleden, fellows en arts-assistenten van de ICK die tijdens de beoordeling van ieder kind aan het rijtje A – B – C – D – de "R" van "Research" hebben toegevoegd om op ieder tijdstip van de dag de onderzoekers op de hoogte te brengen van nieuwe opnames of behandelingen.

Mijn promotor, Prof. dr. Tibboel, beste Dick, toen ik als student op uw deur klopte waar de sleutels nog aan de buitenkant in zaten, vertelde u mij stellig *"Lex, je kan nog 26 jaar met een pieper op zak lopen, waarom zo'n haast?"* waarna ik achter u aan moest rennen de ICK op voor een leerzame casus. Dit is het moment waarop ik mijzelf realiseerde dat er meer is dan *dokter worden*. Hier ben ik u nog steeds dankbaar voor. Al snel leerde ik dat *–de deur met de sleutels er nog in–* letterlijk en figuurlijk altijd open staat om van gedachten te wisselen over onderzoek maar ook over goede vakantiebestemmingen. Ik bewonder uw gedrevenheid en enthousiasme voor de wetenschap en ben daarom meer dan trots dat u mijn promotor bent.

Mijn twee copromotoren. Dr. de Wildt, beste Saskia, gepassioneerd als je de farmacologie bespreking en de hele kinderfarmacologie-onderzoekslijn in het leven hebt geroepen, alsmede de toewijding waarmee je 's nachts tijdens je dienst toch nog een abstract voorziet van scherpe kritieken, heb ik bijzonder gewaardeerd. Maar, bovenal heb ik genoten van de gezelligheid *buiten* het Sophia; hoeveel promovendi kunnen zeggen dat ze met hun copromotor gaan borrelen, curlen, of wielrennen langs de Maasvlakte? Volgende uitje richting de ark *–zonder muizen–* in Friesland?

Dr. Cransberg, beste Karlien, in 2010 stond ik op de stoep als student zonder enige "nefrologische bagage". Het moet dan ook een hele opgave zijn geweest om mij het nodige te leren. Desondanks ging je deze strijd vol vertrouwen aan. Jouw toewijding aan zogezegd "de nieren" de afgelopen vier jaar, is dan ook onmisbaar geweest voor het project. De cappuccino's & Belgische chocolade die je in de weekenden langs bracht, waren onmisbaar voor mij. Een dokter met hart voor je patiënten en promovendi, zorgzaam en geïnteresseerd in een ander als altijd, bedankt.

De leden van de kleine promotiecommissie, Prof. dr. van den Anker, Prof. dr. van der Heijden, en Prof. dr. Reiss wil ik hartelijk danken voor het beoordelen van mijn proefschrift. Prof. van den Anker, beste John, hartelijk dank voor uw gastvrijheid in Washington DC, het was een onvergetelijke en leerzame ervaring. Prof. van der Heijden, altijd belangstel-

lend naar de voortgang van de AKI-studie waarvoor hartelijk dank. Prof. Reiss, beste Irwin, eerst op de ICK en nu op de ICN, eindeloos enthousiast bereid wetenschap of kliniek met een lach te bediscussiëren, hetgeen ik zeer heb gewaardeerd. Prof. dr. van Gelder, prof. dr. Gommers, en prof. dr. de Hoog wil ik hartelijk danken voor het zitting nemen in de grote commissie.

Alle co-auteurs wil ik bedanken voor de prettige samenwerking in de afgelopen jaren.

Dr. Yolanda de Rijke en Monique de Waart, bedankt voor jullie expertise in de diverse biomarker bepalingen. AKC laboranten, Barry en Paul in het bijzonder, dank voor de honderden urine en bloed samples die zijn verdeeld over duizenden cupjes.

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Beste Ko Hagoort, of je nu in Indonesië of het Sophia verblijft, je bereidheid om ten allen tijde een manuscript of discussie te reviseren zijn prijzenswaardig. Monique van Dijk, ondanks dat het een 'AKI' boekje is geworden heb ik jouw persoonlijke betrokkenheid en zorgzaamheid altijd erg gewaardeerd. Hanneke IJsselstijn, jouw standaard *–binnen-10-minuten–* reply's maakte onze samenwerking een genot. Saskia Gischler, oprecht en heerlijk recht door zee.

De ICK staf, 24/7 paraat, en desondanks vriendelijk en behulpzaam, bedankt voor ieders support. In het bijzonder, Enno, sparren over een studie-opzet of sample procedure, ECMO-vraagstukken of een kriebelhoest op congres oplossen, fysiologisch denken; ik heb veel van je geleerd. En ja.. ik ben inmiddels besmet met het ECMO-virus waarvoor dank! Ulrike, Sara, hoe druk de kliniek ook is, steevast tijd voor een praatje of advies, dank.

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Joke Dunk: rots in de branding! Samen tot middernacht met bevroren vingers -80 samples sorteren, of een database *met spoed* vullen, op jouw steun kon ik blindelings vertrouwen, mijn dank is groot. Marianne Maliepaard, geen PRISM of PIM is jouw teveel.

Dear Prof. Ray, dear Patricio, you made me feel really welcome at Children's National Medical Center. Thank you for the opportunity to experience true laboratory research. Remember, even though the Argentinian soccer team won in '78, our King still married an Argentinian Queen! Dear Sophia, thank you for your help with and guidance in all the biomarker assays, I truly enjoyed it.

Dear Jan, my American Mom, thank you for your hospitality. Staying with you and your family in Washington DC was an unforgettable experience. Thanksgiving dinner was my first and best ever.

Tijdens mijn onderzoeksperiode heb ik op meer bureaus mogen werken dan ik op twee handen kan tellen. Ondanks de vele verhuizingen die enige organisatie vereisten, resulteerde mijn zwerftocht door het Sophia in contact met een inspirerende mix aan individuen, variërend van chirurg tot collega-promovendi, van research nurse tot de portier.

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SP-1324; Dit was nog eens goed vertoeven! Een bank, die goed van pas kwam tijdens de nachten op de ICK, *Nespresso… what else?* en uiteraard: veel, heel veel gelachen. MC, ouwe, bedankt voor je steun, en niet te vergeten heerlijke Brabantse gezelligheid met *–hoe kan het ook anders–* worstenbroodjes. Ik bewonder jouw toewijding voor het vak chirurgie; of het nou longen wordt of GE, ik weet zeker dat je jouw dromen nastreeft. Nienke, jouw nuchterheid is goud waard. Heel veel succes met de laatste loodjes, je zit tenslotte op een "schrijfstoel" en je weet het: je bent er bijnaaaa! Bram, ouwe anesthesist, ondanks dat ik soms hard moest lachen om je lijstjes moet ik eerlijk bekennen dat je de meest gedisciplineerde en tevens sociale persoon bent die ik ken. Je kan heerlijk

gevat uit de hoek komen, en staat werkelijk voor iedereen klaar: een unieke combinatie. Erik, hoeveel uren wij wel niet op de ICK hebben doorgebracht?? Al metend of samples verzamelend. Ondanks dat de batterij soms leeg was *–en ik dus genoodzaakt was een opname op het moment suprême af te spelen in plaats van op te nemen…–* heb ik met veel plezier onze diensten gedraaid. Neuroloog, ik wist het! Gerbrich, AIOS (yes!), zanger Rinus, lachen gieren brullen tijdens de vele meetings en etentjes. Ondanks dat ik je heb leren kennen als collega, kletsen we werkelijk over alles behalve werk, bedankt.

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Diederick, Died, eerst IC en nu samen promoveren binnen het Erasmus. We hebben Doppio de afgelopen jaren goed gesponsord. Lieve Ritu, al vanaf de peuterspeelzaal vieren wij samen onze verjaardag, het maakt niet uit waar of wanneer, dat zegt genoeg! Ik waardeer het dan ook erg je er vandaag bij bent.

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Bas, aller-aller-liefste, meer woorden heb ik niet nodig: *We're gonna rock the Rockies!*