Detection of acute kidney injury in premature asphyxiated neonates by serum neutrophil gelatinase-associated lipocalin (sNGAL) – sensitivity and specificity of a potential new biomarker

Biljana Pejović*[1], Jelena Erić-Marinković[2], Marija Pejović[3], Jelena Kotur-Stevuljević[4], Amira Peco-Antić[5]

[1]Institute of Neonatology, Neonatal Intensive Care Unit, Belgrade, Serbia
[2]School of Medicine, Institute of Medical Statistics and Informatics, University of Belgrade, Belgrade, Serbia
[3]School of Medicine, University of Belgrade, Belgrade, Serbia
[4]Faculty of Pharmacy, University of Belgrade, Belgrade, Serbia
[5]School of Medicine, University of Belgrade, University Children's Hospital, Department of Nephrology, Belgrade, Serbia

*Corresponding author: biljapejovic@gmail.com

Abstract

Introduction: Acute kidney injury (AKI) is common in neonatal intensive care units (NICU). In recent years, every effort is made for early detection of AKI. Our hypothesis was that serum neutrophil gelatinase-associated lipocalin (sNGAL) may be a reliable screening test for early diagnosis of AKI in premature neonates after perinatal asphyxia. Therefore, our aim was to assess the diagnostic accuracy of sNGAL for AKI in premature asphyxiated neonates.

Materials and methods: AKI was defined in the third day of life (DOL 3) as a serum creatinine (sCr) increase $\geq$ 26.5 µmol/L from baseline (the lowest previous sCr). According to the increase of sCr, AKI patients were divided in AKIN1 (sCr increase up to 1.9 baseline) and AKIN2 (sCr increase from 2.0 to 2.9 baseline). sNGAL levels were measured on DOL 1, 3 and 7.

Results: AKI was diagnosed in 73 (0.676) of 108 enrolled premature asphyxiated neonates. Sixty one patients (0.836) were classified in AKIN1 and 12 patients (0.164) in AKIN2. sNGAL reached the maximal concentrations on DOL 1 within 4 hours after admission to NICU, being higher in AKI compared with no-AKI group (160.8 $\pm$ 113.1 vs. 87.1 $\pm$ 81.6; $P < 0.001$) as well as in AKIN2 compared with AKIN1 group (222.8 $\pm$ 112.9 vs. 147.8 $\pm$ 109.9; $P < 0.001$). The best areas under the receiver operating characteristic curves (AUC) for prediction of AKI were 0.72 [95% (0.62-0.80) $P < 0.001$] on DOL1 at 2h and 0.72 [95% (0.63-0.80) $P < 0.001$] at 4th hour after admission respectively. The corresponding sNGAL cutoff concentrations were 84.87 ng/mL (sensitivity 69.0% and specificity 71.9%) and 89.43 ng/mL (sensitivity 65.7% and specificity 74.3%).

Conclusions: In premature asphyxiated neonates sNGAL measured within the first 4 hours of DOL 1 is predictive of the occurrence and severity of AKI. Therefore, plasma levels of NGAL may be used for early diagnosis of AKI in these patients.

Key words: serum neutrophil gelatinase-associated lipocalin; acute kidney injury; premature neonates; biomarker

Received: April 16, 2015 *Accepted: September 09, 2015*

Introduction

Incidence of acute kidney injury (AKI) is the highest during the first month of life as newborn's kidneys are more susceptible to injury (1-3). The incidence rate for AKI in the Institute for neonatology in Belgrade, where this study was performed has been estimated to be about 30% of newly admitted newborns *per* year. AKI is common complication in asphyxiated neonates with prevalence up to 70% in the neonatal intensive care units (NICU) (1,2).

The current practice in the diagnosis of AKI is based on conventional biomarkers, serum creatinine (sCr) and urine output which are rather late and unreliable AKI markers (2,3). This diagnosis of AKI in newborns can be problematic due to many factors (3). During the first 48-72 h of life sCr still

reflects maternal levels (3-5). Further, in preterm infants with very low glomerular filtration rate, normal sCr concentrations vary greatly, depending on the level of prematurity and age (3-6). Furthermore, the diagnosis of AKI is delayed as sCr may not be changed until 25-50% of the kidney function has already been lost (3). Additionally, if Jaffe assay is used for sCr quantification, the results may be influenced by medications and hyperbilirubinemia (3). Finally, at lower renal function, sCr will overestimate renal function due to tubular secretion of creatinine (3). Oliguria, defined as urine output less than 1 mL / kg / h (3), is considered as an alternative or additional marker of AKI. We did not use urinary output for diagnosis of AKI. In neonates, urinary output is unhelpful test as non-oliguric renal failure might be present in majority of cases (7).

The delayed AKI diagnosis has an impact on the maintenance of high rates of AKI mortality in NICU (3,6). Therefore, great efforts are being made to find new biomarkers that enable early AKI diagnosis, within a few hours after the onset of kidney damage (3,6,8). One of the most promising AKI biomarker is neutrophil gelatinase-associated lipocalin (NGAL) which is actually not so new (8-13).

Diagnostic concentration of serum NGAL (sNGAL) has not yet been sufficiently investigated in premature neonates with perinatal asphyxia (PNA) (13).

Hypothesis of the present study was that serum neutrophil gelatinase-associated lipocalin (sNGAL) is a reliable screening test for early diagnosis of AKI in premature neonates after perinatal asphyxia. Therefore, our aim was to assess the diagnostic accuracy of sNGAL for AKI in premature asphyxiated neonates.

Material and methods

Subjects

This cross-sectional diagnostic accuracy study was performed from January 2009 to December 2012 in NICU of Institute for neonatology in Belgrade which is representative of the severe ill neonatal population in Serbia with approximately 980 annual rates of patients.

Over the study period, there were 653 admissions of premature neonates with perinatal asphyxia to the NICU from which a total 108 patients were recruited into the study (Figure 1). The premature neonates were included consecutively only if informed consent was given and were admitted within one hour of birth with diagnosis of perinatal asphyxia that was set up by the experienced neonatologist. Perinatal asphyxia (PNA) was defined as a failure to initiate sustained breathing at birth plus Apgar score (AS) of less than 7 in the 5th minute (14).

Five hundred fifty patients were screened for neonatal and maternal excluding reasons. Three hundred eighty neonates with one or more of the following findings were excluded from the study: known renal or any other congenital anomalies, sepsis, metabolic disease, hyperbilirubinemia, re-

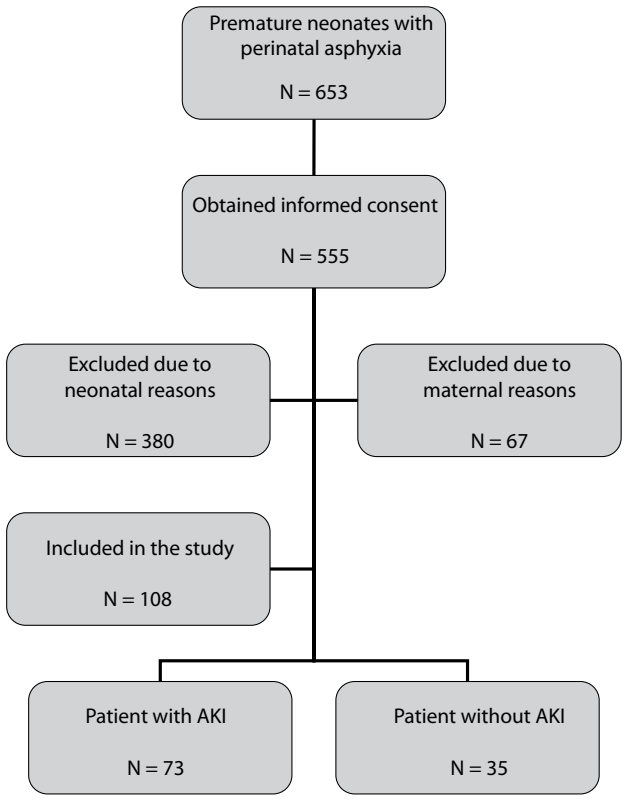

Figure 1. Flow chart showing the patient recruitment process.

ceiving diuretics and other drugs that can affect kidney (the first 48h), or if they did not survive beyond the first 3 days of life.

The purpose of the above listed analyses was to evaluate the health status of patients and that in the case of sepsis, hyperbilirubinemia, or existing renal disease they would be excluded from the study. The study lasted for a long time due to strictly adherence to inclusion and exclusion criteria which ruled out a large number of premature children with asphyxia from recruitment into the study (Figure 1). Eighty seven newborns whose mother had chronic kidney disease, hypertension, severe systemic disease, diabetes mellitus or some other chronic disease were also excluded from the research to eliminate the adverse impact of their illness on infant stress and kidney function. Data on the health of mothers were obtained through retrospective review of medical records of Gynecology and Obstetrics Hospitals. Gestational age (GA) was determined by calculation from the last menstrual period and/or antenatal ultrasonography and was confirmed by Ballard score in the postnatal period (15). Hypoxic ischemic encephalopathy (HIE) was defined according to Saranat and Saranat (16). AKI was defined according to the contemporary definition modified for neonates, similar to Kidney Disease: Improving Global Outcome (KDIGO) AKI definition by which a sCr rise by ≥ 26.5 μmol/L (that is equal to 0.3 mg/dl) from baseline defines AKI in the third day of life (DOL 3). The lowest previous sCr serves as the baseline sCr (17,18). AKI patients were subsequently discriminated according to the increase of sCr into AKIN1 (sCr increase up to 1.9 baseline) and AKIN2 (sCr increase from 2.0 to 2.9 baseline) groups.

Methods

Laboratory investigations, including complete blood count, C-reactive protein, blood urea nitrogen, sCr and bilirubin were determined daily. Complete blood count and C-reactive protein were measured from venous whole blood, using analyzer ABX Micros CRP200, Horiba, France. Urea and bilirubin were measured from serum samples: urea on Maxmat analyzer using Maxmat reagents (Maxmat S.A., Montellier, France), while bilirubin was determined on bilirubinometer Reichert Unistat (Depew, NY, USA). Blood pH and base deficit (BD) were taken daily from capillary blood usually from heel foot and measured on gas analyzer Radiometer, Copenhagen, Denmark. Venous blood samples were taken by micro tube without gel, Eppendorf Safe-Lock Tubes™, 1.5 ml (Eppendorf, Hamburg, Germany), while 125 μL were taken, for blood gas analyses, by capillary tubes with heparin (3-A GLASS, Belgrade, Serbia).

For sNGAL venous blood samples (1.0-1.5 mL) were taken five times: on DOL 1 three times (2, 4 and 6 hours after admission to NICU), and once on DOL 3 and DOL 7. Blood samples were centrifuged at 1000 x g for 10 minutes and the collected serum was stored at -80 ^{0}C until assayed. Analysis of sNGAL was done using human-specific commercially available enzyme-linked immunoassays (ELISA) (R&D Systems, Inc., Minneapolis, Minnesota, USA) according to the manufacturer's recommendations. Concentrations of sNGAL were expressed in ng/mL. Based on our results, analytical accuracy characteristics of the test were as follows: intra-assay coefficient of variation (CV) 3.7%, inter-assay CV 6.5%; linearity from 0.156 ng/mL - 10 ng/mL, with sensitivity of 0.012 ng/mL. We have performed method intra and inter-assay precision determination. The standard concentrations for this analysis were 5 ng/mL, 2.5 ng/mL and 1.25 ng/mL of recombinant human Lipocalin-2 substance (R&D Systems, Inc., Minneapolis, Minnesota, USA). Before every patient's group analysis, we performed analytical pilot study in order to determine the highest possible concentration and the best dilution ratio for the group. We have chosen the 10 samples with the highest sCr concentrations and also find NGAL concentrations in literature on the similar population. We expected the highest concentrations about 300 ng/mL and used 1:20, 1:40, 1:80 and 1:100 dilutions. Dilutions were performed with original R&D Systems Inc. Sample diluents reagent. We got the lowest CVs with 1:80 dilutions and this dilution we have used for further analyses. For those samples which have concentrations below the lowest point at the standard curve, we repeated analysis with lower dilution, 1:40. Serum creatinine concentrations were measured using

Jaffe's method (reagent produced by Human, Wiesbaden, Germany) on a Dimension auto analyzer (Siemens Healthcare GmbH, Germany using reagents Human, Wiesbaden, Germany). Intra-assay CVs was 2.0% and 1.34% for Cr concentrations of 122 μmol/L and 350 μmol/L, respectively. We have performed analyses every six months in order to prevent inter-run variability. Stability of the NGAL according to Han *et al*. (19) is up to one year at -80 ^{0}C and up to 11 months according to Pedersen *et al*. (20).

Laboratory investigators were blinded to clinical outcomes. Urine output was systematically measured by collecting into plastic collecting bags or through a urinary catheter. Diuretics were not given to any patient during the first 48 hours. Oliguria was defined as urine output < 1mL / kg / hour (3). General supportive care was applied according to the NICU´s protocol. The length of mechanical ventilation and the NICU stay were recorded. This clinical and research activities were done according the principles of the Declaration of Helsinki and the study was approved by Ethics committee of the School of Medicine University of Belgrade and Ethics committee of the Institute for Neonatology in Belgrade. Written consent was obtained from parents of the examined children.

Statistical analysis

The Kolmogorov-Smirnov test was used to assess types of distribution of investigated parameters. Normally distributed data were expressed as mean and respective standard deviations, while the non-normally distributed data were expressed as median and corresponding interquartile range. Categorical variables were expressed as frequencies and percentages or relative values. Significance of between-group differences was tested by the parametric independent t-test, nonparametric Mann-Whitney test, and chi-square test where appropriate. To compare sNGAL concentrations among the three groups (no-AKI, AKIN1 and AKIN2) the non-parametric Kruskal-Wallis test was used. To compare sNGAL concentrations trough time (2 hours, 4 hours, 6 hours, day 3 and day 7) in each study group the non-parametric Friedman test has been used. The receiver operating characteristic (ROC) analysis was used to estimate diagnostic accuracy of sNGAL in discriminating between AKI and non-AKI as well as between AKIN1 and AKIN2 neonates, i.e. to determine optimal cut-off concentrations, area under ROC curve (AUC), with respective 95% confidence intervals (95% CI), specificity and sensitivity values, as well as likelihood positive and likelihood negative values along with their 95% confidence intervals. All multivariate analyses were adjusted on the well-known confounders: gestational age, gender, 5-min AS, birth body weight (BBW), and BD of the neonates. There were 5 models for assessing the potential independent effects of sNGAL concentrations in five different time points on AKI presence as well as 5 models for AKIN2 presence. Models are presented with odds ratios and theirs 95% confidence interval along with P.

Statistical analyses were performed using IBM Corp. Realized 2013. IBM Statistics for Windows, Version 22.0 Armonk, NY: IBM. $P < 0.05$ was considered to be statistically significant.

Results

One hundred eight premature neonates of mean GA 34.15 (28.10-37.0) gestational weeks (gw) with BBW of 2214 ± 551 g and AS of 5.0 ± 1.6 were enrolled in study. Sepsis, or other infections or inflammatory status were excluded by normal CRP levels. Patients´ clinical characteristics are summarized in Table 1. Seventy three patients (0.676) were classified in AKI group and the remaining 35 patients (0.324) in no-AKI group. As expected by study design, AS was lower and BD higher in AKI than in no-AKI group. HIE was also more common in AKI than in no-AKI group, as there were a longer period of mechanical ventilation and hospital NICU length of stay in that group. Two lethal outcomes were registered only in AKI group. From AKI patients 0.836 (N = 61) were grouped in AKIN1 and 0.164 (N = 12) in AKIN2 group. Serum NGAL concentrations significantly changed during observation time points as shown in Table 2. They were significantly higher in AKI compared with no-AKI patients on DOL 1 at 2, 4 and 6 hours after admission and in AKIN2 compared with AKIN1 at 4 hours after admission to NICU. Unfortunately, there are

Table 1. Clinical characteristics of the patients.

Characteristics	No-AKI N = 35	AKI N = 73	P	AKIN1 N = 61	AKIN2 N = 12	P
Male/Female***	31/4	35/38	0.911	26/35	9/3	0.812
Gestational age (weeks)*	33.9±1.9	33.9±1.9	0.059	33.8±2.1	34.1±1.8	0.650
Birth body weight (g)**	2195±551	2257±579	0.726	2204±535	2322±627	0.963
Apgar score, 5 min**	6.0 (4.0-7.0)	5.0 (4.0-6.0)	0.039	5.0 (4.0-6.0)	4.5 (3.0-6.8)	0.590
Base deficit (mmol/L)*	7.8±2.5	9.0±3.2	0.016	8.5±3.1	11.6±2.9	0.001
Oliguric patients***	4/35	5/73	0.061	2/61	3/12	0.168
Hypoxic-ischemic encephalopathy***	6/35	35/73	<0.001	25/61	10/12	<0.001
Mechanical ventilation (days)**	2.0 (0.0-3.0)	7.0 (5.0-10.0)	<0.001	7.0 (5.0-9.0)	15.0 (7.2-20.2)	0.002
Hospital NICU length of stay (days)**	6.0 (4.0-7.0)	11.5 (5.0-18.0)	<0.001	10.5 (5.0-18.0)	17.0 (7.0-23.0)	<0.001
Alive on day 10 of life***	35/35	71/73	0.066	61/61	10/12	<0.001
Basal serum Creatinine on DOL1 (μmol/L)**	96 (84-116)	102 (92-122)	0.037	104 (95-132)	92 (83-106)	0.026
Serum Creatinine DOL3 (μmol/L)**	108 (89-118)	160 (138-183)	<0.001	157 (136-174)	189 (170-211)	0.010
Serum Creatinine DOL7 (μmol/L)**	98 (88-138)	134 (100-157)	<0.001	133 (98-155)	156 (123-182)	0.038

DOL - day of life; DOL1 - The first day of life; DOL3 - The third day of life; DOL7 - The seventh day of life; NICU - neonatal intensive care unit; AKI - Acute kidney injury; No-AKI - without AKI; AKIN1 - Stage 1 of AKI; AKIN2 - Stage 2 of AKI.
*mean ± standard deviation; **Median (Interquartile- IQR range); ***ratio.

Table 2. sNGAL in the premature neonates with and without acute kidney injury from day 1 to day 7.

sNGAL	No-AKI Median (IQR)	AKIN1 Median (IQR)	AKIN2 Median (IQR)	P#
$DOL1_2$	66.6 (24.7-100.8)	98.9 (61.8-245.7)	128.0 (93.4-259.4)	0.001
$DOL1_4$	71.5 (28.2-98.4)	98.8 (70.6-224.0)	216.7 (113.3-340.1)	<0.001
$DOL1_6$	68.1 (39.3-117.2)	108.6 (64.3-208.2)	151.5 (91.4-345.9)	0.003
DOL3	74.3 (43.3-104.2)	98.8 (67.3-98.8)	113.8 (90.0-207.4)	0.025
DOL7	78.5 (30.2-98.5)	80.1 (50.4-128.7)	107.1 (75.9-173.8)	0.055
P##	0.002	<0.001	0.002	

Data are presented as median and interquartile range (IQR).
sNGAL - serum neutrophil gelatinase-associated lipocalin concentration; AKI - acute kidney injury; no-AKI - without AKI; AKIN1 - Stage 1 of AKI; AKIN2 - Stage 2 of AKI; DOL - day of life; $DOL1_2$ - 2 hours after admission in DOL1; $DOL1_4$ - 4 hours after admission in DOL1; $DOL1_6$ - 6 hours after admission in DOL1; DOL3 - The third day of life; DOL7 - The seventh day of life.
according to Kruskal-Wallis test; ## according to Friedman test.

some missing results due to technical reasons (Table 3). The results of the diagnostic accuracy of sNGAL in study time periods evaluated by non-parametric ROC curves are presented in Tables 3 and 4. To predict AKI AUC was highest on DOL1 within the first 4 hours after admission to NICU, being even more significant when adjusted on known (or generated in this study with $P \leq 0.10$) confounders such as gender, GA, 5-min AS, BBW, and BD. The best AUC for prediction of AKI were 0.72

Table 3. Serum NGAL predictive accuracy for AKI and its severity.

	sNGAL (ng/mL)	N	AUC	(95% CI)	Optimal cutoff	Sensitivity (%) (95% CI)	Specificiy (%) (95% CI)	LR+ (95% CI)	LR- (95% CI)
no-AKI vs. AKI	$DOL1_2$	103	0.72	(0.62-0.80)	84.9	69.00 (56.9-79.5)	71.90 (53.3-86.3)	2.45 (1.9-3.2)	0.43 (0.2-0.8)
	$DOL1_4$	108	0.72	(80.63 -0.80)	89.4	65.75 (53.7-76.5)	74.29 (56.7-87.5)	2.56 (2.0-3.3)	0.46 (0.2-0.9)
	$DOL1_6$	106	0.68	(0.58-0.76)	54.2	88.90 (79.3-95.1)	44.10 (27.2-62.1)	1.59 (1.1-2.3)	0.25 (0.1-0.5)
	DOL3	107	0.63	(0.53-0.72)	106.5	45.83 (34.0-58.0)	82.86 (66.4-93.4)	2.67 (2.0-3.6)	0.65 (0.3-1.4)
	DOL7	107	0.59	(0.49-0.69)	38.2	88.89 (79.3-95.1)	34.29 (19.2-52.2)	1.35 (0.8-2.2)	0.32 (0.2-0.6)
no-AKI vs. AKIN 1	$DOL1_2$	91	0.70	(0.59-0.81)	84.9	64.41 (34.9-90.1)	71.87 (57-9-83.6)	2.29 (0.7-8.7)	0.50 (0.2-1.4)
	$DOL1_4$	96	0.69	(0.58-0.80)	89.4	60.66 (34.9-90.1)	74.29 (62.7-85.5)	2.36 (1.3-4.2)	0.53 (0.2-1.2)
	$DOL1_6$	95	0.66	(0.54-0.78)	54.2	86.89 (48.2-97.7)	74.12 (39.0-94.0)	2.55 (0.9-6.3)	0.5 (0.1-1.8)
	DOL3	95	0.60	(0.48-0.72)	100.5	43.33 (15.2-72.3)	82.86 (69.6-90.5)	2.53 (0.7-8.9)	0.68 (0.3-1.6)
	DOL7	95	0.56	(0.44-0.69)	38.2	86.67 (51.6-97.9)	34.29 23.1-48.4)	1.32 (0.9-2.0)	0.59 (0.2-1.6)
AKIN1 vs. AKIN 2	$DOL1_2$	71	0.61	(0.49-0.73)	88.0	91.67 (61.5-97.9)	45.76 (33.7-60.0)	1.69 (1.1-2.5)	0.18 (0.03-1.2)
	$DOL1_4$	73	0.72	(0.60-0.82)	102.7	91.67 (61.5-97.9)	54.10 (41.6-67.9)	2.00 (0.6-7.1)	0.15 (0.02-1.0)
	$DOL1_6$	72	0.65	(0.53-0.76)	64.9	100.0 (73.5-100.0)	26.20 (15.8-39.1)	1.36 (0.9-2.1)	0.00
	DOL3	72	0.64	(0.51-0.75)	69.9	100.0 (73.5-100.0)	33.30 (21.3-46.0)	1.50 (1.1-2.2)	0.00
	DOL7	72	0.65	(0.53-0.76)	91.0	75.00 (42.8-94.5)	60.00 (47.3-72.9)	1.87 (1.1-3.1)	0.42 (0.2-1.2)

sNGAL - serum neutrophil gelatinase-associated lipocalin concentration; AKI - Acute kidney injury; no-AKI - without AKI; AKIN1 - Stage 1 of AKI; AKIN2 - Stage 2 of AKI; DOL - day of life, DOL 1_2 - 2 hours after admission in DOL1; DOL 1_4 - 4 hours after admission in DOL1; DOL 1_6 - 6 hours after admission in DOL1; DOL3 - The third day of life; DOL7 - The seventh day of life; CI - confidence interval; (LR+) - Likelihood ratio positive; (LR-) - Likelihood ratio negative; AUC - area under the receiver-operating-characteristic curve.
According to Hanley-McNeil test.

[95% (0.62-0.80) P < 0.001] on DOL1 at 2 h and 0.72 [95% (0.63-0.80) P < 0.001] at 4 hours after admission, respectively.

The corresponding sNGAL cutoff concentrations were 84.9 (sensitivity 0.690 and specificity 0.719) and 89.4 ng/mL (sensitivity 0.657 and specificity 0.743) (Table 3). A diagnostic concentrations of sNGAL as risk factor for AKI development was also confirmed with multivariate logistic regression analysis showing an increasing risk for AKI with increasing concentrations of sNGAL on DOL 1, 3 and 7 (Table 4).

Table 4. The association between sNGAL concetrations from day 1 to day 7 and development of acute kidney injury.

Dependent variables	sNGAL	Multivariate model#	
		OR	(95 % CI) P
AKI vs. no-AKI	sNGAL DOL 1_2	1.185	(0.000-7.139) 0.999
	sNGAL DOL 1_4	1.012	(1.003-1.020) 0.007
	sNGAL DOL 1_6	1.009	(1.002-1.015) 0.010
	sNGAL DOL 3	1.006	(1.001-1.012) 0.030
	sNGAL DOL 7	1.007	(1.000-1.014) 0.046
AKIN2 vs. AKIN1	sNGAL DOL 1_2	1.003	(0.996-1.010) 0.384
	sNGAL DOL 1_4	1.005	(0.999-1.011) 0.117
	sNGAL DOL 1_6	0.005	(0.998-1.012) 0.194
	sNGAL DOL 3	1.006	(0.997-1.014) 0.191
	sNGAL DOL 7	1.010	(1.000-1.021) 0.055

sNGAL - serum neutrophil gelatinase-associated lipocalin concentration; AKI - Acute kidney injury; no-AKI - without AKI; AKIN1 - Stage 1 of AKI; AKIN2 - Stage 2 of AKI; DOL - day of life; DOL 1_2 - 2 hours after admission in DOL1; DOL 1_4 - 4 hours after admission in DOL1; DOL 1_6 - 6 hours after admission in DOL1; CI - confidence interval; OR - odds ratio. # Adjusted for: gender, gestational age, 5-min Apgar score, birth body weight, base deficit.

Discussion

This study investigated the diagnostic accuracy of sNGAL in preterm neonates with perinatal asphyxia without other risk factors that may have impact on renal function and/or on NGAL concentrations. Plasma NGAL is strongly influenced by inflammation. Therefore, in patients with any inflammation, diagnostic concentrations of sNGAL for the detection of AKI might be restricted. Further studies in NICU setting should develop biomarker panel which in combination with risk stratification, could differentiate patients admitted with sepsis from those admitted for other indications. We planned to include about 100 patients. Given that a large number of patients were not eligible for recruitment due to strict compliance with inclusion and exclusion criteria, the study was extended to the fulfillment of the expected number of the eligible patients. NGAL was tested in serum, so as in urine. However, the scope of this article is devoted only to serum NGAL analysis as urine samples were not obtained in all patients in the first day of life due to delayed micturation after delivery.

We have confirmed our hypothesis that sNGAL may be marker of AKI in asphyxiated premature neonates. Serum NGAL level had a moderate discriminatory power to predict sCr-based AKI with AUC of 0.72. What is also remarkably is that sNGAL may differentiate AKI from no AKI patients within the first 4 hours after perinatal asphyxia. This is very important from the point of the implementation of the early treatment of critically ill premature infants to improve their outcome (3,12). We found also that the risk of AKI occurrence and severity increases with increased concentration of sNGAL. Interestingly, the prediction of AKI using sNGAL AUC was better for AKIN1 than for the whole AKI group that may be attributed to its better sensitivity for earlier stages of AKI. Namely, as found by other authors (9,10,21), it could be expected that predictive values would increase when sNGAL is measured closer to the time of insult.

Our results are in line with those from literature regarding NGAL (9-13,21-28). Since 2003, when Mishra *et al*. pointed importance of NGAL as an early AKI biomarker in children after cardiac surgery (9), number of papers which reported the role of NGAL in predicting AKI has been rapidly in-

creasing (10-13,21-28). Although the follow-up studies failed to show the outstanding performance of the first trials (9), the implementation of NGAL in early AKI diagnosis in previously healthy patients without comorbidities remains very good (24). There are no many pediatric studies which have examined NGAL outside of the cardiopulmonary bypass population and quite a few researchers have investigated the benefit of sNGAL in diagnosis of AKI in newborns. Krawczeski *et al*. examined serum and urine NGAL in 374 infants undergoing cardiopulmonary by-pass (CBP). Only 35 were newborns and 5 (0.143) of them developed AKI. They found that NGAL predicted AKI at 2 hours after CBP with AUC ranged 0.74-0.88 (10). In case control study of Sarafidis *et al*. (13) which included 13 asphyxiated and 22 non-asphyxiated newborns, 8 patients had AKI. Serum NGAL, as well as urine NGAL and serum Cystatin C predicted better PNA than AKI as they were higher in asphyxiated neonates, even in those not developing AKI (13). Ragall *et al*. investigated sNGAL in 30 newborns with HIE of whom 13 (0.433) developed AKI (25). They found elevated sNGAL measured within 6 hours after birth reliably indicates (AUC 0.968) AKI in asphyxiated neonates. Similar results, but with urinary NGAL (uNGAL) were reported by Essajee *et al*. (27). They used uNGAL not only to predict serum creatinine-based AKI in asphyxiated premature neonates, but also to define AKI to associate a Day 1 uNGAL concentration above a specific threshold with clinical outcome (27,28). Finally Smertka *et al*. examined 73 newborns with sepsis and 29 without sepsis (26). Five sepsis patients (0.680) developed AKI. They concluded that NGAL more accurately reflect the severity of inflammatory status than AKI (26). No one of our patients had sepsis or other documented infections or inflammatory status. Therefore, an increase of sNGAL may be attributed only to AKI. We used DOL 3 sCr based definition of AKI having in mind that at that time sCr better reflects the kidney function in newborns. Serum creatinine concentrations measured in the first hours of life which were lower in AKI compared with those in no-AKI group may be explained by lower maternal sCr, and additionally confirms that patients from AKI group had no prenatal kidney damage. Like other authors (7,29) we didn't find urine output to be useful for the diagnosis of AKI, as most of AKI patients were non-oliguric.

The strengths of this study include: first, the evaluation of great population of preterm neonates with PNA; and second, the prospective nature of data collection and rigorous protocol for sampling, which was followed by blind measurements of biomarkers. Third, the examination started in first or second hour of life, promptly after hypoxic-ischemic insult. Finally, early identification with sNGAL of newborns who are at risk for developing AKI, or who already have AKI may contribute to improvement of their treatment and outcome. Despite these strengths, this study has many limitations. The first one is that we used sCr as the gold standard for the diagnosis of AKI, although it is delayed marker of renal function and it does not rise until significant loss of renal function occurs. It is possible that some patients with "negative creatinine, NGAL positive" were misclassified in no-AKI group that could negatively affect the diagnostic accuracy of sNGAL. We are also aware of the limitation of Jaffe reactions to determine sCr. In addition, this study is limited by small simple size being a single-center study that needs validation at the larger group of patients at multicenter level.

Furthermore, our results were obtained in selected premature neonates with mild to moderate degree of perinatal asphyxia without other neonatal and maternal risk factors, and therefore cannot be generalized on complete neonatal population, including term neonates, who are significantly different from preterm ones in response to PNA and in susceptibility of developing AKI. Moreover, patients who died before 3 day of life were not included, but in these patients early diagnosis of AKI is very important. At last, although we have demonstrated that sNGAL may have a role in early stage AKI identification, it is still unclear how much sNGAL incorporation into clinical care will improve AKI outcome. Regarding that, the ELISA tests for sNGAL determination that we have used (because we do not have any other test for some automated analyzer system) are not suitable for urgent parameters determination, because this is long-term and time-consuming method. We hope that

health diagnostics manufacturer will develop some kind of immunochemical reagents which could be applied at automatic analyzer system.

Conclusions

In premature asphyxiated neonates sNGAL measured within the first 4 hours of DOL 1 is predictive of the occurrence and severity of AKI. Therefore, plasma levels of NGAL may be used for early diagnosis of AKI in these patients. By allowing earlier timing of injury and earlier intervention, it could improve AKI outcome.

Acknowledgements

Financial support for this study was provided by the Ministry of Education, Science and Technological Development Republic of Serbia, grant No 175 079.

Potential conflict of interest

None declared.

References

1. *Shah P, Riphagen S, Beyene J, Perlman M. Multiorgan dysfunction in infants with post-asphyxial hypoxic-ischaemic encephalopathy. Arch Dis Child Fetal Neonatal Ed 2004;89:F152–5. http://dx.doi.org/10.1136/adc.2002.023093.*
2. *Andreoli SP. Acute renal failure in the newborn. Semin Perinatol 2004;28:112-23. http://dx.doi.org/10.1053/j.semperi.2003.11.003.*
3. *Askenazi DJ, Ambalavanan N, Goldstein SL. Acute kidney injury in critically ill newborns: what do we know? What do we need to learn? Pediatr Nephrol 2009;24:265-74. http://dx.doi.org/10.1007/s00467-008-1060-2.*
4. *Kaur S, Jain S, Saha A, Chawla D, Parmar VR, Basu S, Kaur J. Evaluation of glomerular and tubular renal function in neonates with birth asphyxia. Ann Trop Paediatr 2011;31:129-34. http://dx.doi.org/10.1179/146532811X12925735813922.*
5. *Gallini F, Maggio L, Romagnoli C, Marrocco G, Tortorolo G. Progression of renal function in preterm neonates with gestational age < or = 32 weeks. Pediatr Nephrol 2000;15:119-24. http://dx.doi.org/10.1007/s004670000356.*
6. *Askenazi DJ, Griffin R, McGwin G, Carlo W, Ambalavanan N. Acute kidney injury is indepedently associated with mortality in very low birthweight infants: a matched casecontrol analysis. Pediatr Nephrol 2009;24:991-7. http://dx.doi.org/10.1007/s00467-009-1133-x.*
7. *Karlowicz MG, Adelman RD. Nonoliguric and oliguric acute renal failure in asphyxiated term neonates. Pediatr Nephrol 1995;9:718-22. http://dx.doi.org/10.1007/BF00868721.*
8. *Soni SS, Ronco C, Katz N, Cruz DN. Early diagnosis of acute kidney injury: the promise of novel biomarkers. Blood Purif 2009;28:165–74. http://dx.doi.org/10.1159/000227785.*
9. *Mishra J, Dent C, Tarabishi R, Mitsnefes MM, Ma Q, Kelly C, et al. Neutrophil gelatinase-associated lipocalin (NGAL) as a biomarker for acute renal injury after cardiac surgery. Lancet 2005;365:1231-8. http://dx.doi.org/10.1016/S0140-6736(05)74811-X.*
10. *Krawczeski CD, Woo JG, Wang Y, Bennett MR, Ma Q, Devarajan P. Neutrophil gelatinase-associated lipocalin concentracions predict development of acute kidney injury in neonates and children after cardiopulmonary bypass. J Pediatr 2011;158:1009-15. http://dx.doi.org/10.1016/j.jpeds.2010.12.057.*
11. *Peco-Antić A, Ivanišević I, Vulićević I, Kotur Stevuljević J, Ilić S, Ivanišević J, et al. Biomarkers of acute kidney injury in pediatric cardiac surgery. Clin Biochem 2013;46:1244-51. http://dx.doi.org/10.1016/j.clinbiochem.2013.07.008.*
12. *Lavery AP, Meinzen-Deer JK, Anderson E, Ma Q, Bennett MR, Devarajan P, Schibler KR. Urinary NGAL in premature infants. Pediatr Res 2008;64:423-8. http://dx.doi.org/10.1203/PDR.0b013e318181b3b2.*
13. *Sarafidis K, Tsepkentzi E, Agakidou E, Diamanti E, Taparkou A, Soubasi V, et al. Serum and urine acute kidney injury biomarkers in asphyxiated neonates. Pediatr Nephrol 2012;27:1575-82. http://dx.doi.org/10.1007/s00467-012-2162-4.*
14. *World Health Organization. Basic newborn resuscitation: a practical guide (1998). Available at: http//www.who.int/maternal_child_adolescent/documents/who_rht_msm_981/en/. Accessed September 1st, 2015.*
15. *Ballard JL, Novak KK, Driver M. A simplified score for assessment of fetal maturation of newly born infants. J Pediatr 1979;95:769-74. http://dx.doi.org/10.1016/S0022-3476(79)80734-9.*
16. *Sarnat HB, Sarnat MS. Neonatal encephalopathy following fetal distress: a clinical and electroencephalographic study. Arch Neurol 1976;33:696-705. http://dx.doi.org/10.1001/archneur.1976.00500100030012.*
17. *Kidney Disease: Improving Global Outcomes (KDIGO) Acute Kidney Injury Work Group. KDIGO Clinical Practice Guideline for Acute Kidney Injury. Kidney Int Supplement 2012;2:1–138.*

18. Saeidi B, Koralkar R, Griffin RL, Halloran B, Ambalavanan N, Askenazi DJ. Impact of gestational age, sex, and postnatal age on urine biomarkers in premature neonates. Pediatr Nephrol 2015; [Epub ahead of print]. http://dx.doi.org/10.1007/s00467-015-3129-z.
19. Han WK, Wagener G, Zhu Y, Wang S, Lee HT. Urinary biomarkers in the early detection of acute kidney injury after cardiac surgery. Clin J Am Soc Nephrol 2009;4:873–82. http://dx.doi.org/10.2215/CJN.04810908.
20. Pedersen KR, Ravn HB, Hjortdal VE, Rregaard RN, Povlsen JV. Neutrophil gelatinase-associated lipocalin (NGAL): validation of commercially available ELISA. Scand J Clin Lab Invest 2010;70:374–82. http://dx.doi.org/10.3109/00365513.2010.486868.
21. Wasung ME, Chawla LS, Madero M. Biomarkers of renal function, which and when? Clin Chim Acta 2014;438:350-7. http://dx.doi.org/10.1016/j.cca.2014.08.039.
22. Hsu CW, Symons JM. Acute kidney injury: can we improve prognosis? Pediatr Nephrol 2010;25:2401-12. http://dx.doi.org/10.1007/s00467-010-1508-z.
23. Sweetman DU, Molly EJ. Biomarkers of acute kidney injury in neonatal encephalopathy. Eur J Pediatr 2013;172:305-16. http://dx.doi.org/10.1007/s00431-012-1890-6.
24. Mårtensson J, Bellomo R. The rise and fall of NGAL in acute kidney injury. Blood Purif 2014;37:304-10. http://dx.doi.org/10.1159/000364937.
25. Raggal NE, Khafagy SM, Mahmoud NH, Beltagy SE. Serum neutrophyl gelatinase-associated lipocalin as a marker of acute kidney injury in asphyxiated neonates. Indian Pediatr 2013;50:459-62. http://dx.doi.org/10.1007/s13312-013-0153-6.
26. Smertka M, Wroblewska J, Suchojad A, Majcherczyk M, Jadamus-Niebroj D, Owsianka-Podlesny T, et al. Serum and urinary NGAL in septic newborns. Biomed Res Int 2014;2014:717318. http://dx.doi.org/10.1155/2014/717318.
27. Essajee F, Were F, Admani B. Urine neutrophil gelatinose associated lipocalin in asphyxiated neonates: A prospective cohort study. Pediatr Nephrol 2015;30:1189-96. http://dx.doi.org/10.1007/s00467-014-3035-9.
28. Goldstein SL. Urinary NGAL to define AKI in asphyxiated infants. Pediatr Nephrol 2015;30:1047-9. http://dx.doi.org/10.1007/s00467-015-3046-1.
29. Bezerra CT, Vaz Cunha LC, Libório AB. Defining reduced urine output in neonatal ICU: importance for mortality and acute kidney injury classification. Nephrol Dial Transplant 2013;28:901-9. http://dx.doi.org/10.1093/ndt/gfs604.