Evaluation of Novel Biomarkers for Early Diagnosis of Acute Kidney Injury in Asphyxiated Full-Term Newborns: A Case-Control Study

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Significance of the Study

- Previous studies have focused on urinary parameters, marker proteins or renal indices, but a holistic study combining all three has been rarely embarked upon.
- We explored the individual and combined ability of serum cystatin C, beta 2-microglobulin, urinary neutrophil gelatinase-associated lipocalin, and alpha 1-microglobulin to predict acute kidney injury in asphyxiated neonates.
- This study provides a reference for combined detection of renal injury after neonatal asphyxia.

Keywords

Acute kidney injury · Asphyxia · Full-term neonates · Urinary neutrophil gelatinase-associated lipocalin

Abstract

Objectives: To investigate the changes of serum cystatin C (Cys-C), beta 2-microglobulin (β 2-MG), urinary neutrophil gelatinase-associated lipocalin (NGAL), and alpha 1-microglobulin (α 1-MG) in asphyxiated neonates, and to evaluate the value of combined detection of multiple biomarkers in the early diagnosis of acute kidney injury (AKI) in asphyxiated neonates. **Methods:** A total of 110 full-term asphyxiated and 30 healthy neonates were included. The asphyxia neonates were divided into AKI and non-AKI groups. Serum Cys-C, β 2-MG, urine NGAL, and α 1-MG were measured 24 h after

birth. The diagnostic value of the biomarkers was determined using receiver operating characteristic (ROC) curves.

Results: There was no significant difference in serum creatinine and blood urea nitrogen among the control group, moderate asphyxia group, and severe asphyxia group at 24 h after birth. Significant differences were noticed in terms of serum Cys-C, β 2-MG, urinary NGAL, and α 1-MG among the 3 groups. Moreover, with the aggravation of asphyxia, the above indicators gradually increased. There were significant differences in the 4 indicators between the AKI and non-AKI groups ($p < 0.05$). The area under the ROC curve of the above indicators was 0.670, 0.689, 0.865, and 0.617, respectively ($p < 0.05$). The sensitivity and specificity of the combined diagnosis of asphyxia neonatorum AKI with the 4 indicators were 0.974 and 0.506, respectively. **Conclusions:** Serum Cys-C, β 2-MG, urine NGAL, and α 1-MG are early specific indicators for

the diagnosis of renal injury after neonatal asphyxia. Combined detection of these parameters could aid clinical evaluation of renal injury in asphyxiated neonates.

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Introduction

Asphyxia is an important cause of morbidity and mortality among neonates. The incidence of asphyxia is estimated to be 10 per 1,000 live births and is influenced by the local availability of medical resources [1]. It can lead to multi-organ dysfunction and a redistribution of cardiac output to maintain cerebral, cardiac, and adrenal perfusion while potentially compromising renal, gastrointestinal, and skin perfusion [2]. Renal injury in asphyxiated preterm infants has a high incidence (30–55%) and mortality rate 60–66% [3]. Renal impairment in perinatal asphyxia has been noted by several studies in the past [4, 5]. However, diagnosis of acute kidney injury (AKI) is difficult in neonates as many of the established clinical and biochemical parameters are unreliable in this age group [6]. The immediate and long-term outcome depends to a large extent on the early recognition and appropriate management of complications.

Most renal function markers commonly used in clinical practice assess glomerular function. These include creatinine (Cr)-based parameters such as serum creatinine (sCr), from which the glomerular filtration rate (GFR) or endogenous Cr clearance is calculated, as well as measures of GFR calculated from serum cystatin C (Cys-C) [7]. Human Cys-C is a low-molecular weight protein, belonging to the cystatin superfamily of protease inhibitors, which is produced at a constant rate in all nucleated cells. Cys-C is freely filtered through the glomerular membrane, then completely reabsorbed and degraded by the proximal tubule. Serum Cys-C is being promoted as a more accurate estimate of neonatal GFR [8]. Serum beta 2-microglobulin (β 2-MG) is of special interest to us, because it has been associated with the risk of end-stage kidney disease and death [9]. It belongs to low-molecular weight proteins that are freely filtered via the glomerular membrane and catabolized in tubules [10]. Urinary concentration of β 2-MG is a sensitive index of renal tubular function in asphyxiated neonates [11]. AKI biomarkers in the urine have been shown to be predictive of AKI and mortality in children undergoing cardiopulmonary bypass [12] and in critically ill preterm infants [13]. Urinary neutrophil gelatinase-associated lipocalin (NGAL) has bacteriostatic

properties and contributes to innate immunity [14]. At the early phase of ischemia-induced AKI there is a rapid upregulation of NGAL mRNA in the Henle loop and proximal tubules, causing an increase in the synthesis and excretion of NGAL into the urine. In normal conditions, serum NGAL is filtered by the glomerulus and rapidly reabsorbed by the proximal tubule [15]. There is evidence that it can serve as a useful marker in pediatric populations with high predictive efficiency [16]. A previous study has suggested that the urinary measurement of alpha 1-microglobulin (α 1-MG) can be a useful method of screening populations in whom there is a risk of tubular proteinuria whatever the underlying cause [17].

The ideal marker for detecting AKI should be upregulated shortly after an injury and independent of the level of GFR [18]. sCr levels and changes in urine output are the most commonly applied measures of renal function; however, using Cr to monitor renal function and to diagnose AKI is not ideal for many reasons: (i) the Cr value in a neonate reflects maternal Cr; (ii) Cr is a measure of function (not injury), and it is a late marker of an acute injury; (iii) >50% of nephrons must be compromised before changes in the Cr level become evident, so it is a late marker of significant renal dysfunction; (iv) at lower GFR, serum Cr will overestimate renal function, owing to tubular secretion of Cr; (v) Cr varies by muscle mass, hydration status, age, and gender; and (vi) bilirubin and medications can affect Cr measurement by the Jaffe method [19].

Previous studies have focused on urinary parameters, marker proteins, or renal indices but a holistic study combining all of these has been embarked upon. To investigate the utility of serum and urine AKI biomarkers in asphyxiated neonates, we evaluated 6 previously identified candidate serum and urinary biomarkers: sCr, blood urea nitrogen (BUN), serum Cys-C, β 2-MG, urinary NGAL, and α 1-MG. We explored the individual and combined ability of these biomarkers to predict AKI in asphyxiated neonates.

Materials and Methods

Patients

A total of 110 asphyxiated full-term newborns admitted in our hospital, between September 2016 and August 2017, were enrolled in our study. Thirty cases of normal newborn infants born in the same period were enrolled as healthy controls. All infants underwent physical examination and laboratory examination.

Inclusion criteria were based on the diagnostic criteria for neonatal asphyxia established by the Neonatal Resuscitation Group of the Chinese Medical Association Perinatal Medicine Branch in 2016. These criteria included infants born at term with perinatal asphyxia as evidenced by 3 or more of the following: (a) cord blood

Table 1. General clinical data of patients with asphyxia and healthy controls

	Healthy controls (<i>n</i> = 30)	Moderate asphyxia (<i>n</i> = 85)	Severe asphyxia (<i>n</i> = 25)	X ² /F	<i>p</i> value
Male/female, <i>n</i>	16/14	58/27	16/9	2.146	0.342
Gestational age, weeks	39.09±1.21	39.30±1.23	39.30±1.52	0.304	0.738
Birth weight, g	3,266±463.15	3,319±456.84	3,349±386.99	0.255	0.775
Birth length, cm	49.17±1.82	49.73±0.95	49.55±1.32	2.237	0.111
Data are presented as means ± SD unless otherwise indicated					

pH <7.0; (b) Apgar score <6 at 5 min; (c) meconium-stained liquor; and (d) changes in fetal heart rate [11].

Neonates were excluded if they had one or more of the following features: (a) prematurity or postmaturity; (b) neonates whose mothers had kidney disease, renal insufficiency, or who have received nephrotoxic drugs like aminoglycosides; (c) and neonates with significant illness (e.g., severe infection, jaundice, primary glomerular diseases such as nephritis), congenital malformations, or who were receiving drugs (e.g., aminoglycosides) which may affect the renal function.

Term normal appropriate for gestational age newborns delivered during the study period matched for hours after birth and sex were taken as controls from the postnatal ward of the same hospital. Gestational age of all the neonates was determined from the date of the last menstrual period of the mother and modified Ballard's scoring system.

The asphyxiated neonates were further grouped into moderate and severe asphyxia on the basis of Apgar score and umbilical cord pH: moderate asphyxia – Apgar score of 1 or 5 min ≤7 points and an umbilical cord pH <7.2; severe asphyxia – Apgar score of 1 min ≤3 points and/or Apgar score of 5 min ≤5 points and an umbilical cord pH <7.0.

Detection Index and Methods

Venous blood (1–3 mL) was collected at 24 h of life for the estimation of blood Cys-C and β2-MG. Urine samples (10 mL) were simultaneously collected using commercially available pediatric urine bags. Care was taken to prevent leakage and contamination of urine with stool. Specimens were preserved at low temperature after centrifugation (venous blood: 3,800 rpm for 8 min; urine sample: 1,800 rpm for 5 min), and then sent to the laboratory. sCr, Cys-C, β2-MG, BUN, urinary NGAL, and α1-MG were measured using an automatic biochemistry analyzer (Cobas 8000 Modular Analyzer Series C701; Roche, Mannheim, Germany).

The precision, accuracy, linearity, and anti-jamming performances were evaluated during the tests on a fully automated biochemical analyzer. sCr and BUN were measured by enzyme methods. A good accuracy was obtained for sCr, with an intraspecific coefficient of variation (CV) of ≤0.8% and an interspecific CV of ≤2.0%. Good reproducibility of standard duplicates was obtained for BUN, with an average signal confidence of variability of 6.5%. Blood Cys-C concentration was determined by latex-enhanced immunoturbidimetric assay with a detection interval between 0.40 and 6.80 mg/L. β2-MG levels were measured using an end-point immunoturbidimetric assay, which employs a polyclonal latex-coated rabbit anti-human β2-MG antibody, on the Roche Modular P Analyzer. The

assay has traceability to the 1st International Standard Preparation (1986, WHO) (Roche Tina-Quant Serum β2-MG Assay Kit, Cat. No. 11660551). The lowest tested concentration with a CV ≤20% was taken as the limit of quantification. The lower reporting limit was taken as the lowest linear concentration whose CV was <20%.

Urinary NGAL was measured by the Neutrophil Gelatinase-Associated Lipocalin Assay Kit by Latex using the enhanced immunoturbidimetry method. A good accuracy was obtained, with an intraspecific CV of ≤5% and an interspecific CV of ≤10%. The measured values were linearly related to the theoretical values when the concentrations of NGAL were in the range of 25–3,000 ng/mL (*r* ≥ 0.990). α1-MG was measured by an immunoturbidimetric assay. A good accuracy was obtained, with an intraspecific CV of ≤10% and an interspecific CV of ≤10%. The measured values were linearly related to the theoretical values when the concentrations of NGAL were in the range of 6–110 mg/L (*r* ≥ 0.990).

The diagnostic criteria for AKI in asphyxiated neonates were as follows: reduction in kidney function within 48 h (currently defined as an absolute increase in sCr of ≥0.3 mg/dL [≥26.4 μmol/L]), a percentage increase in sCr of ≥50% (1.5-fold from baseline), or a reduction in urine output (documented oliguria of <0.5 mL/kg/h for more than 6 h) [20].

Statistical Analysis

The normality of the data was evaluated by the Kolmogorov-Smirnov test. Continuous data with normal distribution were presented as means ± SD and compared using the Student *t* test. Variables with non-normal distribution were expressed as median (interquartile range) and compared using the Mann-Whitney test. Qualitative data were expressed as numbers or percentages and compared using the χ² test. Statistical analysis was performed by SPSS 19.0 software (SPSS Inc., USA). Analysis of diagnostic susceptibility and specificity of β2-MG, Cys-C, α1-MG, and NGAL were realized using the receiver operating characteristic curve (ROC) and the area under the curve (AUC). *p* < 0.05 was considered statistically significant.

Results

Baseline Characteristics

A total of 110 asphyxiated full-term newborns (85 cases in the moderate asphyxia group and 25 cases in the severe asphyxia group) and 30 cases of normal newborn infants were enrolled in our study. The baseline characteristics of

Table 2. Details of the AKI and Non-AKI groups

	AKI (<i>n</i> = 37)	Non-AKI (<i>n</i> = 73)	X ² /F	<i>p</i> value
Male/female, <i>n</i>	29/8	45/28	3.123	0.077
Gestational age, weeks	39.54±1.22	39.18±1.32	-1.416	0.160
Birth weight, g	3,322±408.76	3,328±458.34	0.076	0.939
Birth length, cm	49.77±1.26	49.84±0.83	-0.349	0.728

Data are presented as means ± SD unless otherwise indicated.

Table 3. Levels of biomarkers in asphyxia and healthy groups 24 h after birth

Groups	sCr, μmol/L	BUN, mmol/L	β2-MG, mg/L	Cys-C, mg/L	NGAL, μg/L	α1-MG, mg/L
Healthy controls (<i>n</i> = 30)	49.25±7.42	3.35±1.16	2.25±0.31	1.00±0.20	59±12	8.95 (4.25)
Moderate asphyxia (<i>n</i> = 85)	51.03±9.44	3.89±1.27	2.89±0.84	1.28±0.26	108±31	36.6 (31.90)
Severe asphyxia (<i>n</i> = 25)	52.74±8.58	4.01±1.38	3.54±0.98	1.81±0.39	268±57	68.70 (7.79)
F/Z	1.057	2.446	18.342	60.125	280.22	9.792
<i>p</i> value	0.350	0.090	0.000	0.000	0.000	0.000

Data are presented as means ± SD or median (IQR). sCr, serum creatinine; Cys-C, cystatin C; β2-MG, beta 2-microglobulin; NGAL, urinary neutrophil gelatinase-associated lipocalin; α1-MG, alpha 1-microglobulin.

Table 4. Levels of biomarkers in the AKI and Non-AKI groups 24 h after birth

Group	sCr, μmol/L	BUN, mmol/L	β2-MG, mg/L	Cys-C, mg/L	NGAL, μg/L	α1-MG, mg/L
AKI (<i>n</i> = 37)	51.36 ±10.7	4.35±1.03	3.70±1.17	1.96±0.34	270±88	53.3 (68.6)
Non-AKI (<i>n</i> = 73)	48.15±9.26	4.06±1.14	3.09±0.60	1.78±0.28	93±52	38.20 (27.90)
<i>t</i> /Z	1.625	1.301	-3.588	-2.985	13.247	-2.347
<i>p</i> value	0.107	0.196	0.001	0.004	0.000	0.019

Data are presented as means ± SD or median (IQR). sCr, serum creatinine; Cys-C, cystatin C; β2-MG: beta 2-microglobulin; NGAL, neutrophil gelatinase-associated lipocalin; α1-MG: alpha 1-microglobulin.

the 3 groups are shown in Table 1. No significant differences were observed among the 3 groups in terms of gender, gestational age, birth weight, and birth length ($p > 0.05$).

Meanwhile, 110 asphyxiated full-term newborns were divided into the AKI group and the non-AKI group according to the diagnostic criteria for AKI in asphyxiated neonates [20]. The baseline characteristics in the AKI and non-AKI groups are shown in Table 2. Differences in gender, gestational age, birth weight, and birth length had no statistical significance ($p > 0.05$).

Biomarker Differences in Asphyxia Infants and Healthy Controls

There was no significant difference in terms of sCr and BUN among the control group, moderate asphyxia

group, and severe asphyxia group 24 h after birth ($p > 0.05$), while significant differences were noticed in terms of serum Cys-C, β2-MG, urine NGAL, and α1-MG among the 3 groups ($p < 0.05$). The above indicators showed a gradual upward trend with the aggravation of asphyxia (Table 3).

Biomarker Differences in Infants with and without AKI

There were no significant differences in levels of sCr and BUN between the AKI and non-AKI groups, while the levels of Cys-C and β2-MG in blood, and NGAL and α1-MG in urine in the AKI group were significantly higher than those in the non-AKI group ($p < 0.05$; Table 4).

ROC Analysis of Marker Proteins

Using ROC analysis, we calculated the sensitivity and specificity for each marker's ability to detect AKI (Fig. 1). Values for NGAL gave the best diagnostic performance, with an AUC of 0.865. Analysis of Cys-C concentrations yielded an AUC of 0.670, and β 2-MG and α 1-MG achieved a comparable AUC of 0.689 and 0.617, respectively. Table 5 shows the results of the ROC analysis for all markers at 24 h after birth. The sensitivity and specificity of combined diagnosis of AKI in asphyxiated neonates with the above 4 indicators were 0.974 and 0.506, respectively, indicating that the combined detection of multiple biomarkers could be helpful in the clinical evaluation of AKI in asphyxiated neonates.

Discussion

In this study, we evaluated the association of 6 serum and urine biomarkers with AKI in asphyxiated neonates. We found that the levels of Cys-C and β 2-MG in blood, and NGAL and α 1-MG in urine measured 24 h after birth were associated with AKI. These data suggest their potential as biomarkers of AKI.

Perinatal asphyxia is one of the most important causes of neonatal mortality and morbidity. AKI is independently associated with poor outcomes in the critically ill patient. The standard kidney function biomarker, sCr, shows a demonstrable rise in concentration many hours to days after insult to the kidney. Thus, Cr-based AKI diagnosis is likely delayed, rendering treatments to mitigate or prevent AKI ineffective. Neonatal AKI is further confounded by the fact that sCr concentrations in infants actually reflect maternal levels.

The diagnosis of AKI is usually based on changes in sCr, but such measurements are a poor marker of acute deterioration in kidney function and hence biomarkers are gaining importance. Biomarkers like serum Cys-C, urine interleukin-18 (IL-18), urine kidney injury molecule-1, urine NGAL, IL-18, glutathione-S-transferase-pi, and gamma-glutathione-S-transferase have been used in a few studies on AKI [21, 22].

Estimation of urinary β 2-MG as an indicator of proximal renal tubular dysfunction in asphyxiated full-term newborns has been documented [23]. Cys-C is normally filtered freely and is completely reabsorbed and catabolized within the proximal tubule [24]. Urine Cys-C levels increase with structural and functional renal tubular impairment independent of GFR [25]. It is highly predictive of AKI in children and adults who undergo cardiopulmonary bypass surgery and kidney transplantation [26]. Stud-

Fig. 1. ROC curves of blood Cys-C, β 2-MG, urinary NGAL, and α 1-MG in diagnosing AKI after neonatal asphyxia. Cys-C, cystatin C; β 2-MG, beta 2-microglobulin; NGAL, neutrophil gelatinase-associated lipocalin; α 1-MG, alpha 1-microglobulin.

ies on 22 cases of children with burns admitted to hospital within 12 h revealed that there were no significant differences between the sCr level at 1–5 days in the AKI and the non-AKI groups [27]. By contrast, blood and urine NGAL levels increased considerably in the AKI group, which indicated the sensitivity of NGAL for early detection of AKI [27]. More recently, urinary NGAL was shown to be a good predictor of AKI in 108 term asphyxiated neonates with an AUC of 0.724 when a cutoff of 250 ng/mL was used (56% AKI prevalence) [28]. Hoffman et al. [28] included 25 infants with hypoxic-ischemic encephalopathy receiving hypothermia in a study investigating a panel of urinary biomarkers for their ability to predict AKI in critically ill neonates. Previous studies have shown that urinary concentrations of some biomarkers are dependent on gestational age in children without AKI [29]. This might be secondary to the inability of immature tubules to reabsorb these proteins in underdeveloped kidneys. Controlling for this important confounder is necessary to ensure that the associations between urine biomarkers and AKI are not simply a reflection of tubular maturation [22].

This study has several limitations. First, the study is limited by the relatively small number of patients. Therefore, large-scale, head-to-head comparisons of the different biomarkers are required in the future. Another important lim-

Table 5. Analysis of β 2-MG, Cys-C, α 1-MG, and NGAL

	24-h AUC	95% CI	Sensitivity, %	Specificity, %	Cutoff
β 2-MG	0.689	0.586–0.791	0.487	0.787	3.49 mg/L
Cys-C	0.670	0.565–0.774	0.615	0.693	1.86 mg/L
α 1-MG	0.617	0.508–0.726	0.461	0.8	57.35 mg/L
NGAL	0.865	0.788–0.943	0.846	0.880	109.5 μ g/L

Cys-C, cystatin C; β 2-MG, beta 2-microglobulin; NGAL, urinary neutrophil gelatinase-associated lipocalin; α 1-MG, alpha 1-microglobulin.

itation of this study is the possibility that other confounders besides gestational age could explain the association between AKI and these biomarkers. Because of limitations in sample size, we were not able to control for other potential confounders, such as inflammatory changes, birth weight, maternal and infant genetic variations, and others. Our selection criteria led to a heterogeneous population of sick newborns. Studies with larger populations will control for these limitations. Third, newborns with moderate to severe encephalopathy underwent therapeutic hypothermia in our study, and this factor may affect the renal outcome. Lastly, further studies about the association of AKI with respect to gestational age and gender are needed.

Conclusion

Neonatal asphyxia can cause changes in glomerular and tubular function, and more severely asphyxiated neonates are more likely to experience renal failure compared to those with milder asphyxia. Serum Cys-C and β 2-MG, and urine NGAL and α 1-MG levels are early and specific indicators of renal damage after asphyxia. The combined

detection of multiple biomarkers may contribute to clinical evaluation of renal injury in asphyxiated neonates. Because of limitations in sample size and the heterogeneous population of sick newborns, this study was not able to control for other potential confounders. Therefore, a large-scale, head-to-head comparison of serum and urinary levels is required to ascertain which of the two is the more useful test.

Statement of Ethics

This study was approved by the Ethics Committee of the local hospital and informed consent was obtained from guardians of all the participants.

Disclosure Statement

The authors declare that they have no conflict of interest.

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