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Urine neutrophil gelatinase-associated lipocalin in asphyxiated neonates: a prospective cohort study

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ORIGINAL ARTICLE

Urine neutrophil gelatinase-associated lipocalin in asphyxiated neonates: a prospective cohort study

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Abstract
Background Acute kidney injury (AKI) is the most common complication of perinatal asphyxia. Recent research indicates that urine neutrophil gelatinase-associated lipocalin (NGAL) is an early marker for AKI; yet, there is a paucity of data about its use in term neonates with perinatal asphyxia.
Methods A prospective cohort study was conducted on 108 term babies in the new-born unit of Pumwani Maternity Hospital and Kenyatta National Hospital. Urine NGAL and serum creatinine were measured in 108 term asphyxiated neonates on days 1 and 3 of life.
Results One-hundred and eight patients were recruited (male:female 1.4:1). At a cut-off of 250 ng/ml, urine NGAL had an acceptable discriminative capability of predicting AKI (area under the curve 0.724). The sensitivity, specificity, positive and negative predictive value and likelihood ratios were 88, 56, 30, 95 %, 2 and 0.2 respectively. Urine NGAL levels were significantly higher in patients with AKI compared with those without AKI. An NGAL level greater than 250 ng/ml on day 1 was significantly associated with severe hypoxic ischaemic encephalopathy (HIE); odds ratio=8.9 (95 % CI 1.78–37.69) and mortality; odds ratio=8.9 (95 % CI 1.78–37.69).
Conclusion Urine NGAL is a good screening test for the early diagnosis of AKI. It is also a predictor of mortality and severity of HIE in asphyxiated neonates.

Keywords Neutrophil gelatinase-associated lipocalin · Acute kidney injury · Perinatal asphyxia

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Introduction

Acute kidney injury (AKI) has emerged as an important health problem in the neonatal population and the care of a child with renal disease has evolved dramatically over the last few decades. The most common complication of perinatal asphyxia is AKI and the current gold standard for the diagnosis of AKI is based on serum creatinine. With all its shortcomings as a diagnostic test, serum creatinine continues to be the marker of choice in the diagnosis of AKI. Creatinine is a late marker of AKI and therefore the search continues for a new biomarker that can replace this imperfect gold standard test with the sole objective of providing an early and accurate diagnosis of AKI in the new-born and in the population in general.

Most of the prevalence studies that have investigated AKI in new-borns have been single-centre studies and report its existence to be between 17.2 and 61 % [1–4].

The lack of a consensus definition and classification criteria of AKI in neonates is mainly due to the paucity of measurable variables and biochemical markers [5]. This has led to difficulty in accurately estimating the prevalence of AKI in newborns, as different studies have used various definitions and cut-offs of serum creatinine to define AKI. The majority of the neonatal studies use a serum creatinine measurement cut-off of 133 μmol/l.

Serum creatinine is currently the gold standard test for diagnosing AKI; yet, it possesses significant shortcomings and is acknowledged to be an inadequate marker of renal injury. Furthermore, the problem with using creatinine as a measure of AKI in neonates is that serum creatinine in the first 24–48 h reflects maternal creatinine, rendering it an unhelpful test in the immediate diagnosis of AKI in the post-natal period [6].

Overall, glomerular filtration rate (GFR) in neonates, both term and preterm, is very low and there is a wide distribution of normal creatinine values, making it difficult to use to assess renal function [7].

Oliguria, a clinical sign of AKI, is not a consistent finding in neonates with perinatal asphyxia, where over 50 % of cases of AKI may occur in the absence of oliguria [3, 5].

Neutrophil gelatinase-associated lipocalin (NGAL) is a 25-kDa member of the lipocalin family and its small size makes it resistant to degradation [8]. NGAL accumulates in the human kidney cortical tubules, blood and urine at very high levels post-ischaemic and nephrotoxic injury, and is one of the earliest proteins induced after kidney injury. Thus, levels of NGAL significantly rise in the blood and urine soon after AKI [9, 10].

Lavery et al. were the first to investigate the role of urine NGAL in premature infants. The sensitivity and specificity of NGAL in detecting renal impairment by serum creatinine greater than 1.2 mg/dl (106 μmol/l) were 20 and 88 % respectively [11]. The sensitivity of NGAL in detecting AKI by oliguria was poor at 31 %, with a specificity of 90 %. This study concluded that the role of NGAL as an early biomarker for AKI in preterm neonates warranted further investigation.

A nested case–control study (n=9 AKI, n=24 no AKI) was performed by Askenazi et al., comparing eight urine AKI biomarkers in infants with AKI, one of which was NGAL. AKI was defined as a rise in serum creatinine by 27 μmol/l or an elevation of 150 μmol/l persisting for 3 days. The study revealed that for urine NGAL, the area under the curve was 0.68, suggesting the poor discriminative ability of urine NGAL as a predictor of AKI [12].

In a recent study published in August 2012, Sarafidis et al. [13] investigated serum and urine AKI biomarkers in 13 asphyxiated neonates at term (8 with AKI, 5 without AKI). Concerning the urine NGAL findings, it was demonstrated that, compared with controls, significantly higher urine and serum NGAL levels were noted in the asphyxia AKI group than in the non-AKI group. Second, urine NGAL had a significant diagnostic performance as a predictor of AKI on day 1 of life (AUC=0.865, sensitivity=100 %, specificity=83.3 %).

This study aimed to investigate the prevalence of AKI using urine NGAL and to determine the diagnostic utility of urine NGAL in asphyxiated neonates with AKI compared with serum creatinine.

Materials and methods

Study design and population

We conducted a hospital-based prospective cohort study at two new-born units in Pumwani Maternity Hospital and the Kenyatta National Hospital, which is representative of the maternal and new-born population in Nairobi. The study enrolled 108 term neonates and was conducted over a 6-month period.

The sample size used to estimate the prevalence of AKI in neonates with perinatal asphyxia was calculated using Fisher's formula [14], with ±10 % margin of error, assuming a prevalence of 61 % [3]. In this study used a confidence interval of 95 % was used to report findings. The reported confidence interval has a probability of 95 %, including the true population prevalence.

The research and ethics board of all the institutions reviewed and approved the study. Neonates were included in the study only if informed consent had been given and had been admitted at term within 24 h of birth with a diagnosis of perinatal asphyxia. All neonates who were preterm, participating in another study or transferred to another facility before day 3 of life, or who had gross congenital malformations, were excluded from the study.

Neonatal demographic details included gestational age, weight, gender, Apgar score, time of birth, requirement for bag mask ventilation, comorbid conditions, use of any nephrotoxic medication and hypoxic ischaemic encephalopathy (HIE) scoring. Perinatal asphyxia was defined as a failure to initiate sustained breathing at birth plus an Apgar score of less than 7 at 5 min [15]. The Apgar score is a quantitative score, usually measured at 1, 5 and 10 min after birth. The infant's heart rate, respiratory effort, muscle tone, response to stimulation (usually pharyngeal suctioning) and colour are assessed. For each of these five components, assessors award a maximum of two points for normal, one point for poor and 0 points for bad. An Apgar score of less than 7 indicates moderate neuro-/cardiorespiratory depression, and a score of less than 3 indicates severe depression [16].

Acute kidney injury was defined as serum creatinine 133 μmol/l or a percentage increase in serum creatinine of ≥50 % (1.5-fold from the baseline) [1, 3, 17].

Hypoxic ischaemic encephalopathy in this study was defined as acute neurological injury secondary to perinatal asphyxia. Clinically, the assessment was done using the Sarnat classification [18].

Urine samples for NGAL measurement and blood samples for creatinine measurement were collected on days 1 and 3 of life. The urine sample was collected either using a urine bag or if the patient was already catheterised for any purpose or required catheterisation, collection was done via a urinary catheter. For collection via a urine bag the site was cleaned with normal saline and subsequently a urine bag attached; once urine was collected into the bag, it was transferred into a urine container and labelled. The blood sample for serum creatinine was collected simultaneously with the urine sample. Samples were immediately stored in an icebox and transported within 2 h of collection to the Aga Khan University Hospital laboratory for processing.

Serum creatinine assays were carried out immediately using the creatinine Jaffe gen.2 assay. Urine samples, on the other hand, were centrifuged at 1,500×g, 4 °C for 15 min to remove debris and stored in labelled polypropylene tubes at −70 °C for later measurement. All samples were then tested

using the Argutus Medical Human NGAL ELISA kit (BIO92) in one batch at the end of data collection.

Before performing the assay, samples were thawed and brought to room temperature (18–25 °C) and mixed gently. All samples were prepared before starting the assay. NGAL analysis by ELISA was performed using the commercially available assay, which was run according to the manufacturer's protocol. All measurements were run in duplicate to ensure accuracy.

The NGAL concentration was measured at 450 nm wavelength. The mean absorbance for each set of duplicate standards, controls and samples was then calculated. If the individual absorbance differed by more than 15 % from the corresponding mean value, the result was considered suspect and was retested again. A standard curve was created using Sigma Plot 10.0, which generated a good curve fit. The mean absorbance for each standard concentration was plotted on the vertical (Y) axis versus the corresponding concentration on the horizontal (X) axis (logarithmic scale).

According to the manufacturer's protocol, recovery of NGAL in undiluted samples is not 100 %, hence samples were diluted by a factor of 20 for NGAL to be measured accurately. The concentrations read from the standard curve were then multiplied by the dilution factor to attain the final concentration of urine NGAL.

Statistical analysis

The use of descriptive statistics has been applied to decipher the characteristics of the collected data. Categorical data/variables were summarised using proportions, while continuous data/variables were summarised using means or medians along with their standard deviations and mean interquartile ranges respectively

Cut-off for urine NGAL

According to the manufacturer, Argutus Medical Company, the ELISA BIO92 NGAL Kit is a research-based assay; hence, recommended cut-off values were not provided for a positive test. Furthermore, a standard cut-off for NGAL concentration in the detection of AKI has not yet been reported, with all existing studies proposing their own cut-offs. Therefore, this study also determined its own cut-off point using applied statistical methods.

In current practice for the determination of AKI, the gold standard test is the use of serum creatinine. Therefore, a logistic regression model was developed to predict the association between the gold standard and the diagnostic test. The sensitivity and specificity of the association was generated and plotted on a graph, and the point of intersection that gave the best sensitivity and specificity was used as the cut-off point. The diagnostic accuracy of urine NGAL was then tested by generating a conventional ROC curve.

To determine the sensitivity and specificity of urine NGAL in identifying AKI, a 2×2 contingency table was created. The positive and negative likelihood ratio and positive and negative predictive values were also analysed.

As urine NGAL on day 1 and serum creatinine of day 3 were non-normally distributed, comparison between the groups was carried out using the non-parametric Kruskal–Wallis test. In addition, non-normally distributed data were expressed using the median. The odds ratio was measured as a test of association.

Results

One-hundred and eight patients were recruited, of whom 16 died before day 3 of life. Inspection of the collected data showed that 58.3 % of the neonates were male and 41.7 % female (male:female ratio 1.4:1). The median (IQR) gestational age was 38 weeks (38, 40) and the mean (±SD) birth weight was 3,035 g (±399.2). Of the 108 patients, 75 and 24.1 % had moderate and severe asphyxia respectively. HIE scoring was coded into severe and non-severe, the distribution of which was 83.3 and 16.7 % respectively.

Comorbid conditions included neonatal sepsis, respiratory distress and convulsion, proportions of which are shown in Table 1.

Table 1 Description of study population (n=108)

Variable	Frequency
Sex, %	
Male	58.3
Female	41.7
Gestational age, weeks, median (IQR)	38 (38, 40)
Birth weight, g, mean (±SD)	3035 (399.2)
APGAR score, %	
Moderate	75
Severe	24.1
Median APGAR (± SD)	4 (1.4)
HIE, %	
Non-severe	83.3
Severe	16.7
Comorbid condition, %	
Respiratory distress	1.9
Convulsion	27.8
Suspected sepsis	44.4
More than 1	17.6
None	8.3

Determination of diagnostic utility of NGAL on day 1

A logistic regression model was used to predict the association between urine NGAL on day 1 (explanatory/independent variable) and creatinine ≥133 μmol/l (definition of AKI) on day 3 of life. A cut-off value was chosen based on previous studies. A cut-off was preferred rather than analysing it as a linear regression because the cut-off is more likely to be of clinical utility. Sensitivity and specificity were calculated and plotted against each other to determine the point of intersection that would be the appropriate cut-off value (Fig. 1).

Figure 1 shows the sensitivity and specificity of urine NGAL for predicting a creatinine of 133 μmol/l. The point of intersection of sensitivity and specificity was at NGAL concentration 250 ng/ml, which was chosen as a cut-off.

An area under the ROC curve, shown in Fig. 2, was then generated to provide a measure of the discriminative capability of the logistic model. The area under the curve was found to be 0.724, which showed that the chosen NGAL cut-off had an acceptable discrimination.

Prevalence of AKI using urine NGAL ≥250 ng/ml

The cut-off for urine NGAL was determined at 250 ng/ml. Consequently, the prevalence of AKI using urine NGAL was found to be 56 % (60 out of 108; 95 % CI 46.6–65.1 %). Of the 56 % of the patients with AKI, 67 % (40 out of 60) were male and 33.3 % (20 out of 60) were female. In contrast, in the non-AKI group 47 % were male and 53 % were female. A 2× 2 contingency table was then created to determine the sensitivity, specificity, positive and negative predictive value, the likelihood ratio and the odds ratio for urine NGAL against the gold standard. This is given in Table 2.

Table 3 shows the diagnostic utility of urine NGAL as derived from Table 2. The calculated sensitivity and specificity are 88 and 56 % respectively. A positive likelihood ratio of 2 suggests

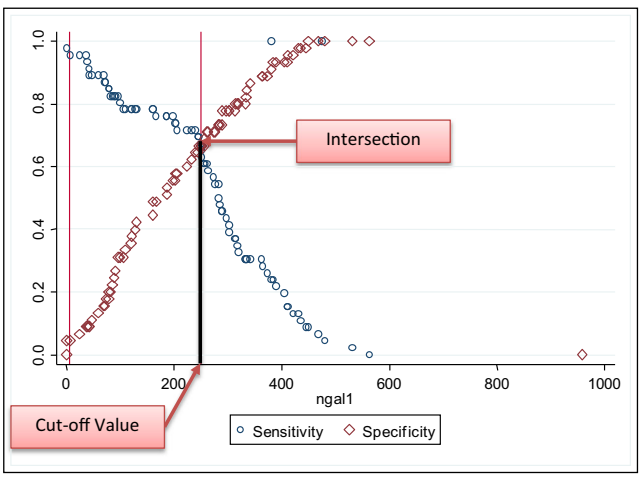

Fig. 1 Graph of sensitivity vs specificity of urine neutrophil gelatinase-associated lipocalin (NGAL)

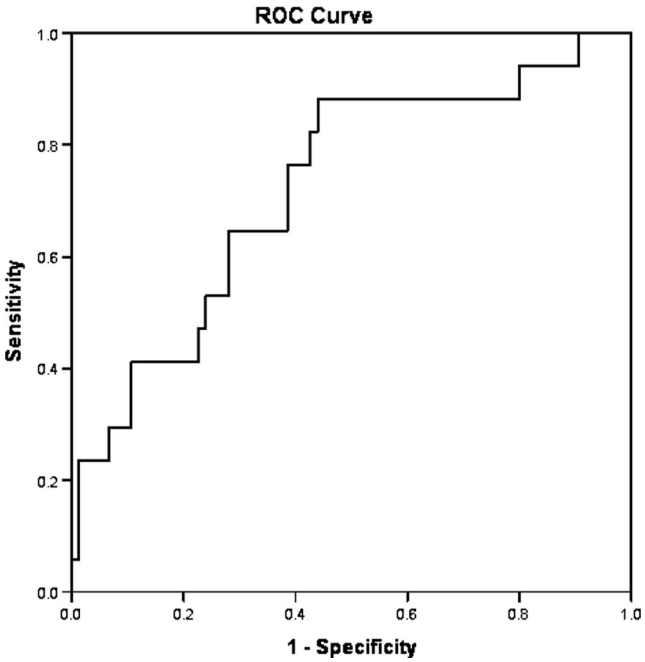

Fig. 2 Receiver operating characteristic (ROC) curve for urine neutrophil gelatinase-associated lipocalin (NGAL) on day 1

that if NGAL is ≥250 ng/ml, the likelihood of having AKI is higher, whilst the negative likelihood ratio of 0.2 suggests that if NGAL is <250 ng/ml the patient is highly unlikely to have AKI.

Comparison of survivors and non-survivors

The 16 patients who died had a significantly higher median for urine NGAL (day 1) 399 ng/ml (IQR 296–448) compared with those who survived: Kruskal–Wallis, Chi-squared = 12.7, $p<0.004$. When comparing this with serum creatinine (day 3), patients who died also had a significantly higher median serum creatinine of 147.5 μmol/l (IQR 134.5–211.5) compared with those who were alive: Kruskal–Wallis, Chi-squared=7.185, $p<0.0073$.

Comparison of severe HIE and non-severe HIE

Patients with severe HIE also had a significantly higher median NGAL (day 1), 391.9 ng/ml (IQR 296–445) compared

Table 2 2×2 Contingency table for urine NGAL vs the gold standard

		Gold standard (serum creatinine 133 μmol/l), day 3		
		No AKI	AKI	Total
Urine NGAL (≥250 ng/ml = AKI; day 1)	No-AKI	42	2	44
	AKI	33	15	48
	Total	75	17	92

NGAL neutrophil gelatinase-associated lipocalin, *AKI* acute kidney injury

Table 3 Results of the diagnostic tests on urine neutrophil gelatinase-associated lipocalin (NGAL)

Test	Result	95 % confidence interval
Sensitivity (%)	88	63.5–98.2
Specificity (%)	56	44.1–67.5
Positive predictive value (%)	31	18.7–46.3
Negative predictive value (%)	95	84.5–99.3
Positive likelihood ratio	2.0	1.4–2.7
Negative likelihood ratio	0.2	0.06–0.8

with those with non-severe HIE: Kruskal–Wallis, Chi-squared =14.5, $p<0.0007$. However, median serum creatinine for patients with severe HIE, 115.5 μmol/l (IQR 85–147.5) was not significantly different from those without severe HIE: Kruskal–Wallis, Chi-squared =2.7, $p<0.255$.

Tests of association

An NGAL level greater than 250 ng/ml on day 1 was significantly associated with severe HIE; odds ratio=8.9 (95 % CI 1.78–37.69). Similarly, a urine NGAL level greater than 250 ng/ml on day 1 was significantly associated with mortality: odds ratio=8.9 (95 % CI 1.78–37.69). In comparison with urine NGAL, it was observed that creatinine levels on day 3 greater or equal to 133 μmol/l were not significantly associated with severity of HIE, maternal and neonatal comorbid conditions except for outcome $p<0.03$. On the contrary, urine NGAL levels greater than 250 ng/ml on day 1 were not significantly associated with any neonatal or maternal comorbid condition ($p<0.961$ and 0.32 respectively).

Comparison of urine NGAL between days 1 and 3

On day 1 of life, significantly higher urine NGAL levels were noted in patients with AKI compared with those without AKI. The levels of NGAL on day 3 remained persistently higher. The box plots in Fig. 3 summarise this. The first box plot shows levels on day 1 of patients with and without AKI. The second box plot shows the same information for day 3.

Discussion

This study investigated the prevalence of AKI in term neonates with perinatal asphyxia. Using urine NGAL, AKI was present in 60 of the 108 (56 %) infants with asphyxia.

The existing studies report an AKI prevalence of 17.2–61 % using serum creatinine in term neonates with perinatal asphyxia. However, because no universal criteria or classification exists to define AKI in the new-born, it is difficult to compare the prevalence between different regions and also amongst different studies. All studies have used various cut-off levels of serum creatinine ranging from 90 to 133 μmol/l to define AKI.

For instance, this study used a diagnostic cut-off for serum creatinine of 133 μmol/l at day 3. Using this cut-off, only 19.5 % of the patients had elevated creatinine >133 μmol/l. However, if the cut-off was reduced to 90 μmol/l, as used in another local African study, the prevalence would increase to 57.6 %. Therefore, in a particular study the prevalence of AKI can be increased by lowering the creatinine cut-off for the diagnosis of AKI. This makes it difficult to appreciate and compare the true disease burden between regions owing to the lack of a universal definition of AKI in new-borns. In our study we therefore opted to measure the prevalence of AKI using urine NGAL on day 1.

Eighty-eight per cent of patients with severe HIE developed AKI using urine NGAL as the biomarker. On the contrary, in non-severe HIE, AKI was present in only 16.6 % of patients. Similar findings of a higher occurrence (61 %) of AKI amongst severely asphyxiated neonates were reported in a study carried out in the USA [3].

The results from this study show that the overall prevalence of AKI is higher in boys than girls, with a ratio of 2.3:1 respectively. These findings are analogous to what has been

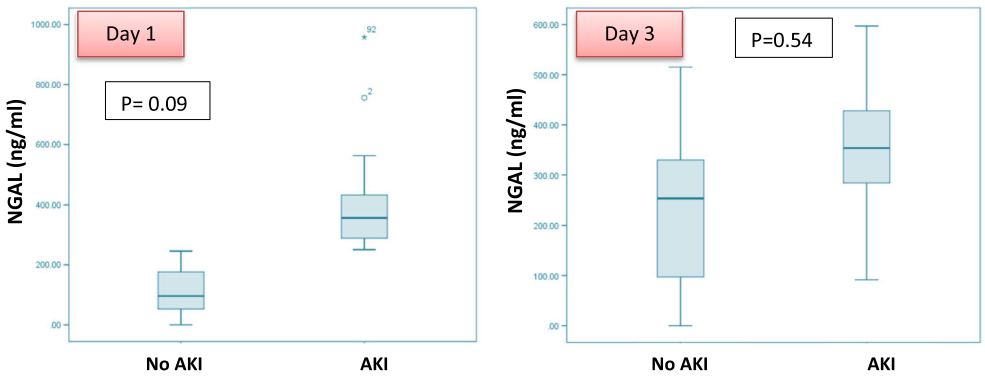

Fig. 3 Box plots of neutrophil gelatinase-associated lipocalin (NGAL) levels on days 1 and 3. *AKI* acute kidney injury

reported in other studies for gender distribution of AKI in the new-born. Nigerian and Iranian studies [19, 20] indicated a male-to-female ratio of 3.3:1 and 2:1 respectively. A probable reason for this pattern could be explained by an increased susceptibility of perinatal disorders in the male new-born population [20]. In contrast, in the non-AKI group the reverse was found, with a male-to-female ratio of 1:1.12.

Urine NGAL has been studied in different population groups as a novel biomarker for the early detection of AKI, with over 400 published articles from animal models to human studies. However, few studies have been conducted using urine NGAL as an early biomarker of kidney injury in neonates. Several cut-off points ranging from 80 to 550 ng/ml have been used to determine the best sensitivity and specificity of urine NGAL [21, 22].

The sensitivity and specificity of NGAL in detecting renal impairment, as indicated by serum creatinine greater than 133 μmol/l, were 88 and 56 % respectively, with an AUC of 0.724. The aforementioned findings are very similar to a meta-analysis of urine NGAL, displaying pooled data for research-based assays. It showed a sensitivity of 76.9 %, a specificity of 83.4 %, AUC of 0.732 and an NGAL cut-off of 246.9 μmol/l [23].

Various studies have also shown that in research-based assays higher variability for cut-off and lower specificity prevail, as is the case in this present study. To what extent these cut-off values concur is currently unknown and requires further investigation.

The few studies carried out in neonates show that urine NGAL has moderate to excellent efficacy as a predictor of AKI. This study showed an overall moderate efficacy (AUC 0.724) in predicting AKI. The differences in these studies could be related to the number of patients enrolled, the prevalence of the disease and whether the study was designed primarily for identifying NGAL levels to diagnose AKI.

The diagnostic performance of a biomarker does not only depend on its ability to detect injury, but also on the disease prevalence and its sensitivity and specificity. The sensitivity of a new biomarker can be reduced from its true sensitivity, especially if the gold standard is not 100 % sensitive or specific. This is true for the present study as creatinine is not 100 % sensitive and specific, thereby affecting the true sensitivity of urine NGAL in this study [24].

Since sensitivity and specificity do not give us the probability that the test will give the correct diagnosis, we calculated the positive and negative predictive values. For the present study, these were 30 % (95 % CI 17.35–44.9) and 95 % (95 % CI 84.5–99.3) respectively. Similar results were seen in a clinical study by Xin et al. [25] on the role of urine NGAL and IL-18 in predicting AKI after surgery. They found that 27.2 % of the patients enrolled in the study had AKI. At a cut-off of 250 ng/ml of urine NGAL, the positive and negative predictive values were 58.3 and 90.5 % respectively.

The positive and negative likelihood ratios for urine NGAL at >250 ng/ml were 2.0 (95 % CI 1.45–2.73) and 0.2 (95 % CI 0.06–0.83) respectively. This means that a positive test result, that is, urine NGAL >250 ng/ml, is twice as likely to be seen in a child with AKI as opposed to one without AKI.

The negative likelihood ratio indicates that any value of urine NGAL <250 ng/ml is associated with the absence of AKI. Our negative likelihood ratio is far away from 1, indicating strong evidence of the absence of AKI if urine NGAL is <250 ng/ml.

Biochemical derangements of urine NGAL on day 1 correlated well with severity of HIE and mortality. The current study revealed significant median levels of urine NGAL for severe HIE and death. Urine NGAL at 250 ng/ml on day 1, correlated well with the severity of HIE and outcome (p= 0.002; odds ratio=8.9). Comparable findings in the literature also support these study findings of urine NGAL as an indicator of mortality [21, 26–28].

No statistically significant association was seen between urine NGAL and the grade of asphyxia. However, when comparing this with the severity of HIE, there was a significant association. This difference can be attributed to the fact that the Apgar scoring is subjective and is not a surrogate marker of AKI, whereas HIE scoring is more objective.

An important finding in this study is how NGAL behaves as AKI progresses. The general trend of NGAL shows similar levels at days 1 and 3, with no statistically significant difference. However, a comparison of levels of urine NGAL on day 1 for patients with and without AKI shows statistically significantly higher levels of NGAL in the former than in the latter. Likewise, a similar trend is seen on day 3. Sarafidis et al. demonstrated that the serum creatinine levels 1 week after birth were similar in those who had an acute rise and those who did not [13]. This means either that the serum creatinine is not sensitive enough to detect complete recovery of kidney function or that the glomerular filtration rate improves faster than tubular repair [7].

There were a number of limitations to the study. A precision error of 10 % was used for sample size estimation instead of the conventional 5 %. This level of precision was adopted because of limitations of resources and study time available. Although serum creatinine is the gold standard, it is not the ideal marker as creatinine provides an estimate of function, not injury, and therefore is not 100 % accurate in detecting AKI. This means that a patient may have AKI, but serum creatinine does not rise until significant loss of function occurs. This present study used serum creatinine as the gold standard for the definition of AKI, which could have hampered the true sensitivity and specificity of urine NGAL, as some patients with evidence of high NGAL, but without any elevation in serum creatinine, were categorised in the non-AKI group. Serum creatinine mystifies early documentation of AKI and lacks the sensitivity for the detection of tubular injury.

Conclusion

This study has shown a relatively high prevalence (56 %) of AKI using urine NGAL, and has also shown that male newborns are affected more than their female counterparts. AKI was more frequently present in babies with severe HIE than in those without.

Urine NGAL is significantly elevated on day 1 in term neonates with AKI compared with those without. Increased urine NGAL concentration is an early marker of AKI. Urine NGAL is also a good screening test owing to its high sensitivity. Likewise, urine NGAL is a significant predictor of mortality and severity of HIE, indicating that this biomarker has both diagnostic and prognostic relevance.

Recommendations

For more accurate prevalence estimation, future definitions of AKI will need improvement with a universal consensus statement and incorporation of clinical end-points, such as mortality and renal replacement therapy.

Further large multicentre prospective randomised controlled trials are required in the neonatal population for the evaluation of urine NGAL as a biomarker for AKI.

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