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Original Article

# Does protein intake correlate with tubular function in very preterm neonates?

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## *Abstract*

***Background*** High protein intake in very preterm neonates (VPN) is important for growth. However, preterm kidneys have fewer functional nephrons and many of the ones present may be immature. Studies have shown that high protein intake induces nephron hypertrophy, proteinuria, and glomerular sclerosis, which lead to tubular injury. Urinary neutrophil gelatinase-associated lipocalin (uNGAL) is a biomarker that is released during proximal tubular cell injury. The uNGAL to creatinine (uNGAL/Cr) ratio is commonly performed for normalization.

***Objective*** To assess for a possible association between protein intake and uNGAL/Cr ratio in VPN.

***Methods*** A prospective cohort study was conducted in two NICUs in Jakarta. Subjects' urine specimens were collected at 0-48 hours, 72 hours, and 21 days after birth to determine uNGAL/Cr ratio as a biomarker of tubular injury. Protein was administered according to study sites NICU guidelines. Protein intake was recorded daily from 14-21 days of age for formula and measured twice with a human milk analyzer for breast milk. ELISA was used to measure uNGAL concentration. Low protein intake was defined as <3g/kg/day and high protein intake was defined as ≥3g/kg/day. Maternal and perinatal variables were recorded from medical records.

***Results*** Fifty-nine VPN were recruited, of whom 39 completed the study. Median uNGAL/Cr ratio ranged from 0.32-104.11 ng/mg. The uNGAL/Cr ratio was not correlated with protein intake but was inversely correlated with gestational age and birth weight [r=-0.320, P=0.019 for the 72-hr (T2) urinary collection]. Higher uNGAL/Cr levels were associated with maternal infection [14.4 (range 4.4-104.1) *vs.* 7.2 (range 0.5-32.4) ng/mg, P=0.004 at the 0-48-hr (T1)], maternal anemia [6.9 (range 1.2-66.6) *vs.* 1.7 (range 0.3-89.2) ng/mg, P=0.001 at the 21-day (T3)] and nephrotoxic medication [15.9 (range 1.3-63.8) *vs.* 1.0 (range 0.4-8.6) ng/mg, P=0.026 at the 72-hr].

***Conclusion*** Protein intake according to current nutritional guidelines does not correlate with tubular injury in VPN, as measured by uNGAL/Cr ratio. Maternal infection, maternal anemia, lower birth weight, and nephrotoxic medication, are associated with higher uNGAL/Cr levels in VPN. **[Paediatr Indones. 2023;63:245-55; DOI: https://doi.org/10.14238/pi63.4.2023.245-55** ].

***Keywords:*** *very preterm neonates; protein; urinary neutrophil gelatinase-associated lipocalin; tubular injury*

Indonesia has the fifth highest rate of preterm birth in the world. In 2014, the preterm birth rate was 10.4%.[1] Our tertiary referral hospital in Jakarta reported an absolute preterm birth rate of 507 in 2018, of whom 112 were very preterm. Very preterm neonates (VPN) are defined as infants born at 28-32 weeks gestational age. The survival rate in our center was 58.9%.[2] The survival rates of VPN have increased as advancements in neonatology care continue. Hence, morbidity caused by organ dysfunction has also increased, including kidney dysfunction.[3]

Very preterm neonates are born with a low number of functional nephrons, which results in reduced glomerular filtration surface area and elevated glomerular arterial pressure.[3,4] Such conditions increase the risk of hypertension and chronic kidney disease (CKD) later in life.[4] Moreover, tubular immaturity, dysfunctional glomerular vasoregulation, low kidney perfusion, renal thrombosis, exposure to nephrotoxic agents, and other events leading to acute kidney injury (AKI) are often found during the care of VPN.[5] It should be remembered that AKI is a

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clinical term, whilst pathology findings describe it as acute tubular injury (ATI). Clinically, AKI is defined as an abrupt increase in serum creatinine level and/or decrease in urine production. Histopathologically, AKI manifests as focal or diffuse tubular luminal dilatation, simplification of the epithelial lining, loss of the brush border in proximal tubules, loss of nuclei, and/or the presence of nucleoli. Proximal and distal tubules may be affected by AKI.[6] Acute kidney injury affected 48% of preterm neonates with 22 to <29 weeks gestational age and 18% of those with 29 to <36 weeks gestational age who participated in the AWAKEN cohort study.[7]

Early tubular injury during AKI does not cause an increase in serum creatinine level, but is detectable by other biomarkers, such as urinary neutrophil gelatinase-associated lipocalin (uNGAL).[8] Expression of uNGAL increases due to decreased reabsorption of NGAL during proximal tubular cell injury. Moreover, de novo synthesis of NGAL increases during AKI in the distal nephron segment, especially in the thick ascending limbs of the loop of Henle and collective ducts, which contributes to the largest fraction of urinary NGAL. The non-invasive method of obtaining urine specimens from neonates for uNGAL examination is beneficial for VPN, since blood sampling is highly restricted due to neonates' low blood volume. In order to control the dilutional factor in urine upon uNGAL testing, the uNGAL to creatinine (uNGAL/Cr) ratio is commonly performed for normalization.[9] In preterm neonates, an uNGAL/Cr cut-off of >100 ng/mg can predict AKI with 89% sensitivity and 33% specificity.[10] Another study in neonates born < 1200 g or < 31 weeks gestational age reported higher median uNGAL/Cr ratio in the AKI group [$6.11x10^6$ (range $3.38x10^6$ - $18.9x10^6$) pg/mg] compared to the non-AKI group [3.17x106 (range $1.59x10^6$ - $6.08x10^6$) pg/mg)]; (P=0.003).[11]

Aggressive nutritional intervention by administering a high protein diet has been used in our NICU to accelerate growth and improve neurodevelopmental outcomes of VPN.[12] This strategy comprises of administering 2.5 g/kg/day protein soon after birth, and gradually increasing to 4 g/kg/day by post-natal age of 3 days.[13] Experimental animal and human studies showed that a high protein diet induces kidney hypertrophy, proteinuria, and glomerulosclerosis via single nephron glomerular hyperfiltration (SNGHF). Hypothetically, SNGHF is an early developmental process of chronic kidney disease in adulthood.[14] Since preterm neonates need high protein intake to catch up to healthy growth percentiles, the impact of high protein intake on preterm nephrons as well as the potential long term effects of this diet requires additional study. A previous study reported that very preterm infants receiving a protein diet of up to 7 g/kg/day showed an increase in estimated glomerular filtration rate (eGFR).[15] However, another study showed that protein intake of up to 3.6 g/kg/day in very preterm infants did not increase the levels of urea, creatinine, serum cystatin C, or urinary β2 microglobulin.[16] The first objective of our study was to analyze for a possible correlation between protein intake and uNGAL/Cr ratio in VPN. The second objective was to assess for possible associations between uNGAL/Cr ratio and prenatal and perinatal factors.

## Methods

This prospective cohort study was performed in 59 VPN from birth to 21 days of age and admitted to the NICU of two referral hospitals, Dr. Cipto Mangunkusumo General Hospital (CMGH) and Bunda Menteng Hospital (BMH), Jakarta, between June 2019 and May 2020. Both hospitals followed the same NICU nutritional guidelines. Participants were consecutively recruited. Infants with major congenital abnormalities, intrauterine growth restriction (IUGR), maternal exposure to nephrotoxic drugs during pregnancy, difficulties in collecting urine specimens, or parental refusal to participate in the study were excluded. Sample size was calculated based on a coefficient correlation formula with an estimated 20% drop out rate.[17] The minimum required sample size was calculated to be 39 subjects.

Spot urine specimens were collected three times during the study, at post-natal age 0-48 hours (T1), 72 hours (T2), and 21 days (T3). These set timings were chosen because neonates were expected to pass their first urine between 0-48 hours. Local guidelines stipulate increasing protein intake from 2.5-3.5 g/kg/day to 4 g/kg/day at 48-72 hours of age, thus, the 72-hour urine collection. Furthermore, the effect of protein in tubular injury was expected after at least

21 days post-exposure of high protein intake. At least 5 mL of urine was collected each time with a urine collector or urethral catheter for uNGAL and urine creatinine measurements. Specimens were kept at 13-15°C for a maximum of 12 hours, then sent to a standardized laboratory in a cooler box at the same temperature. Specimens were centrifuged and then sent for random urine creatinine (uCr) examination. Supernatants were then frozen at -70°C until further analysis. Urinary NGAL was tested by an ELISA method using *QUANTIKINE Immunoassay* (*R&D Systems,* Minneapolis, USA; NGAL Immunoassay). The uNGAL/Cr ratio was calculated by dividing uNGAL (ng/mL) by uCr (mg/dL), and used as a surrogate marker of tubular function.

Protein intake included total parenteral protein in the form of amino acids and oral/enteral protein found in breast milk or formula. The protein intake level was administered according to NICU guidelines for VPN, which was 2.5 g/kg/day of protein in the first 24 hours; 3.5 g/kg/day in the next 24 hours; and 4 g/kg/day thereafter. Neonates were then divided into two groups based on protein intake. Low protein intake was defined as <3g/kg/day and high protein intake was defined as ≥3g/kg/day.[18] Protein intake was recorded from the medical chart at 0-48 hours, 72 hours, and 21 days. From 14 to 21 days of age, the total fluid and total protein intake from both parenteral and enteral sources were recorded. Full feed was defined as consumption of at least 100 mL/kg body weight/day of milk orally/enterally. Enteral diet sources included breast milk, formula milk, and fortified breast milk. Protein intake from formula milk was calculated by dividing the total protein in 100 mL of milk and body weight. Protein intake from breastmilk was calculated by taking the average breastmilk protein content at day 14 and 21. The protein content of breastmilk was measured by testing 5 mL of breast milk in a human milk analyser (*MIRIS*, Uppsala, Sweden; infrared spectroscopy). Protein content from a human milk fortifier (HMF) was also measured based on the volume added to the breastmilk. In VPN with necrotizing enterocolitis (NEC), nutrition including protein was delivered parenterally until clinical improvement was reached.

We recorded basic characteristics of participants who completed the study up to 21 days, such as birth length, birth weight, head circumference, as well as APGAR scores at 1 and 5 minutes. Weight increments were monitored at days 14 and 21 and plotted to an intergrowth post-natal growth (IPNG) curve, producing a weight-to-age *z*-score (WAZ).[19] Maternal factors consisting of infection, anemia, hypertension, and diabetes were recorded. Perinatal factors such as respiratory distress, use and duration of invasive mechanical ventilation (IMV), non-invasive ventilation (NIV), vasoactive medications, and diuretics, nephrotoxic medication exposure, sepsis, necrotizing enterocolitis (NEC), patent ductus arteriosus (PDA), and intraventricular hemorrhage (IVH) were recorded.

Nephrotoxic medication was defined as administration of nephrotoxic agents listed in the *Baby NINJA Study*.[20] In neonatal sepsis cases, aminoglycoside was used for gram negative coverage in combination with beta-lactamase antibiotics. Gentamycin was used as first line and amikacin as second line. Sepsis was diagnosed based on: 1) maternal risk factors (urinary tract and sexually transmitted infections, premature rupture of membranes, intrapartum fever, chorioamnionitis, and/or malodorous discharge); 2) neonatal risk factors (prematurity and low birthweight); 3) hypo-/hyperthermia (temperature <35.5°C or >37.7); 4) respiratory distress; 5) apnea; 6) bradycardia or tachycardia; 7) hypotension; 8) hemodynamic instability; 9) abnormal data: leukocytosis or leukopenia (> 12,000/mm$^3$ or < 4,000/mm$^3$), high CRP, or high I/T ratio regardless of blood culture result.[21] NEC was diagnosed based on modified Bell's staging.[22] PDA was defined as hemodynamically significant shunt between the pulmonary artery and aorta. Hemodynamic significance was diagnosed based on echocardiography showing lung overflow and systemic stealing.[23] The IVH was diagnosed based on head ultrasound.

All statistical analyses were performed using *IBM SPSS Statistics for Macintosh version 21.0,* released in 2012 (*IBM Corp*, Armonk, NY). Clinical characteristics, maternal factors, perinatal factors, and uNGAL/Cr ratio are presented as percentage and median (range) for categorical and numerical data, respectively. The normality test was done using Shapiro-Wilk. Protein intake and uNGAL/Cr ratio at T1, T2, and T3 were analyzed with Spearman's test. Bivariate analysis of uNGAL/Cr

ratio with protein intake, maternal factors, and perinatal factors was performed with Mann-Whitney test. Pearson's correlation was used to analyze uNGAL/Cr with gestational age, birth weight, and APGAR scores at 1 and 5 minutes. The study was conducted in accordance with the *World Medical Association Declaration of Helsinki* and was approved by the Universitas Indonesia Faculty of Medicine Ethics Committee. Informed consent was obtained from parents/caregivers of the participants prior to recruitment.

## Results

A total of 59 participants were recruited. Thirty-nine (66.1%) participants completed the cohort study, but 20 (33.9%) participants died as described in **Figure 1**. Urine specimens were collected consecutively from the 59 VPN at post-natal age 0-48 hours (T1). Of the 59 participants, 53 had a second specimen collected at post-natal age 72 hours (T2), and 39 subjects had a third specimen collected at post-natal age 21 days (T3) in both hospitals, Cipto Mangunkusumo General Hospital (CMGH) and Bunda Menteng Hospital (BMH).

One hundred fifty-one urine specimens were available for analysis. Subjects' characteristics are shown in **Table 1**. Nephrotoxic medication exposure and comorbidities were recorded for CMGH subjects only. Echocardiography and head ultrasound were performed in 44/59 (74.6%) and 41/59 (69.5%) participants, respectively. Median protein intake at the age of 0-48 hours, 72 hours, and 21 days were 2.09 (range 0.72-3.48), 3.12 (range 0.87-4.00), and 3.44 (range 2.00-5.18) g/kg/day, respectively. Median uNGAL/Cr ratios at post-natal age 0-48 hours, 72 hours, and 21 days were 10.37 (range 0.45-104.11), 13.21 (range 0.39- 63.78), 4.22 (range 0.32-89.16) ng/mg, respectively. We found no significant difference in uNGAL/Cr ratios between the high protein intake group and the low protein intake group across all three post-natal ages (**Figure 2**). The uNGAL/Cr ratios were not correlated to protein intake at all times of examination as shown in **Figure 3**. At 0-48 hours uNGAL/Cr showed positive correlation with protein intake, however, the trend at 72 hours and 21 days showed an inverse correlation.

We found maternal infection, hypertension, diabetes, and anemia in 21/57 (36.8%), 17/57 (29.8%), 1/57 (1.8%), and 34/56 (60.7%) of our participants, respectively. Prenatal and perinatal factors related to median uNGAL/Cr at the ages of 0-48 hours, 72 hours, and 21 days are described in **Table 2**. Median uNGAL/Cr was significantly higher in VPN with maternal infection at 0-48 hours (P=0.004) and with maternal anemia at 21 days (P=0.001). The uNGAL/Cr ratio was not associated with maternal hypertension (P>0.05) or maternal diabetes (P>0.05). VPN who developed sepsis and respiratory distress during hospitalization as well as those requiring IMV and NIV support had higher median uNGAL/Cr ratio than VPN without those factors, but the differences were not significant. Additionally, we observed higher median uNGAL/Cr in VPN that were given vasoactive medication, nephrotoxic medication, and diuretics,

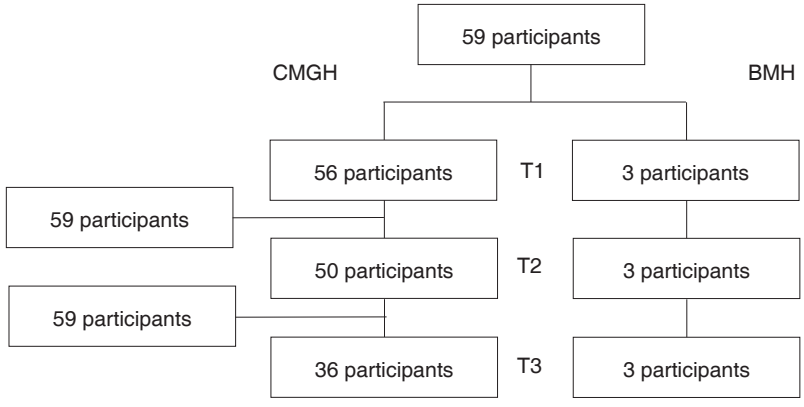


**Figure 1**. Study population

**Table 1**. Subjects' characteristics

| Characteristics | (N=59) |
|---|---|
| Sex, n (%) | |
| Male | 37 (62.7) |
| Female | 22 (37.3) |
| Median birth weight (range), g | 1,240 (795-1800) |
| Median gestational age (range), weeks | 30 (28-32) |
| Median weight increment (range), g/kg/day | |
| Post-natal age 0-14 days[a] | 0 (-13 - 3.2) |
| Post-natal age 0-21 days | 6.5 (-95.4 - 62.9) |
| Median weight-to-age (WAZ) z-score (range) | |
| WAZ at birth | 0.3 (-2.5 - 3.6) |
| WAZ at post-natal age 21 days | 1.3 (-2.9 - 2.8) |
| Nephrotoxic medication, n (%)b | 44 (78.6) |
| NEC, n (%)b | 33 (58.9) |
| PDA, n (%)c | 19 (43.2) |
| IVH, n (%)d | 13 (31.7) |

NEC=necrotizing enterocolitis; PDA=patent ductus arteriosus; IVH=intraventricular haemorrhage; [a]n=45, [b]n=56; [c]n=44; [d]n=41

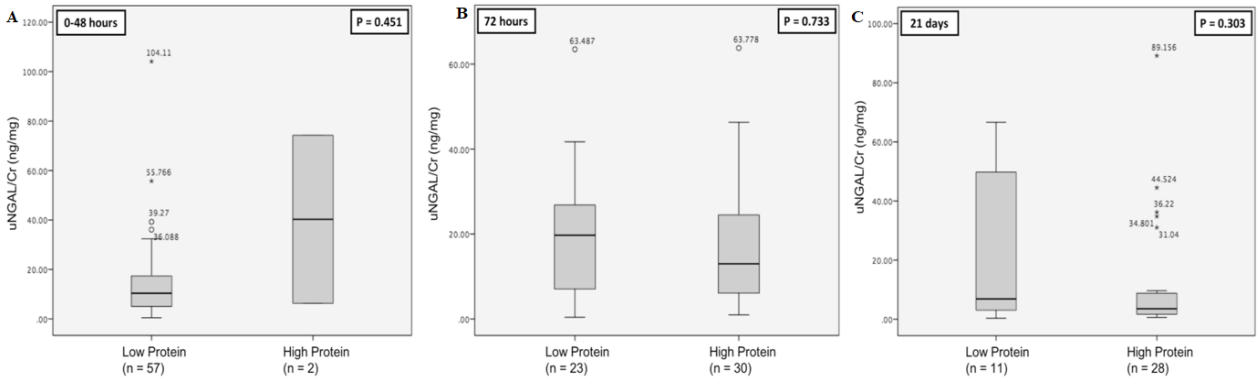


**Figure 2**. Boxplots of NGAL/Cr based on protein level at 0-48 hours, 72 hours and 21 days. There was no significant difference in NGAL/Cr levels between neonates with low (<3 g/kg/day) and high (≥3 g/kg/day) protein intake at (A) 0-48 hours, (B) 72 hours and (C) 21 days. (*P value obtained with Mann-Whitney).

although the only statistically significant association was in those exposed to nephrotoxic medication for uNGAL/Cr ratio at 72 hours (P=0.026). Pearson's correlation was performed to assess for possible correlations between uNGAL/Cr and gestational age, birth weight, as well as APGAR scores at 1 and 5 minutes (**Table 3**). VPN with higher gestational age and higher birth weight had lower uNGAL/Cr levels at all three timepoints, but higher birth weight and lower uNGAL/Cr ratio at 72 hours was the only significant association (P=0.019). Median APGAR score was 6 (range: 2-8) at 1 minute and 8 (range 5-10) at 5 minutes. We found no correlation between uNGAL/Cr level and APGAR score at 1 and 5 minutes.

## Discussion

Our study found no correlation between protein intake and uNGAL/Cr ratios as a biomarker for tubular injury at post-natal ages 48 hours, 72 hours, and 21 days, as shown in **Figure 3**. In the first 48 hours of life, some VPN received a protein intake concentration that was lower compared to the guideline, with a median of 2.09

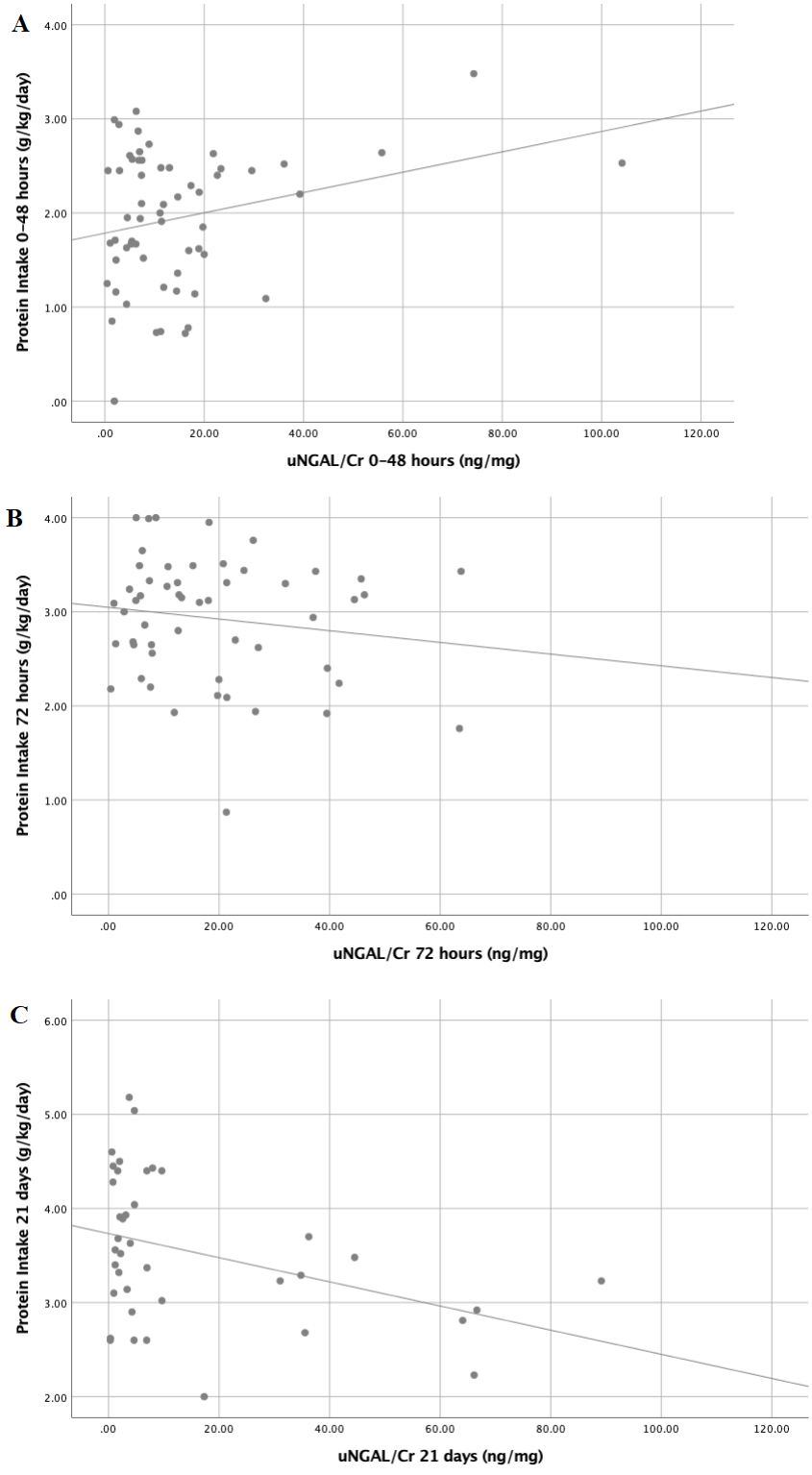


**Figure 3.** Urinary NGAL/Cr ratio with protein intake. The uNGAL/Cr ratio was not correlated with protein intake at post-natal age (A) 0-48 hours, (B) 72 hours, and (C) 21 days.

**Table 2**. Prenatal and perinatal factors associated with uNGAL/Cr level (ng/mg)

| Factors | Median uNGAL/Cr (range)[a] | | | | P value[b] |
|---|---|---|---|---|---|
| | n | Yes | n | No | |
| **Prenatal factors** | | | | | |
| Maternal infection | | | | | |
| 0-48 hours | 21 | 14.4 (4.4-104.1) | 36 | 7.2 (0.5-32.4) | 0.004 |
| 72 hours | 19 | 19.7 (4.4-63.8) | 32 | 12.3 (0.4-44.5) | 0.070 |
| 21 days | 16 | 3.4 (0.6-66.6) | 22 | 4.4 (0.3-89.2) | 0.595 |
| Maternal hypertension | | | | | |
| 0-48 hours | 17 | 7.4 (0.6-18.1) | 40 | 11.3 (0.5-104.1) | 0.105 |
| 72 hours | 15 | 12.7 (1.3-37.5) | 36 | 16.6 (0.4-63.8) | 0.679 |
| 21 days | 10 | 4.4 (0.8-64.1) | 28 | 3.8 (0.3-89.2) | 0.883 |
| Maternal diabetes | | | | | |
| 0-48 hours | 1 | 11.3 | 56 | 9.6 (0.5-104.1) | 0.903 |
| 72 hours | 1 | 5.6 | 50 | 14.2 (0.4-63.8) | 0.277 |
| 21 days | 1 | 64.1 | 37 | 3.9 (0.3-89.2) | 0.158 |
| Maternal anemia | | | | | |
| 0-48 hours | 34 | 11.2 (0.6-55.8) | 22 | 7.2 (0.5-104.1) | 0.421 |
| 72 hours | 28 | 15.9 (2.8-63.5) | 22 | 12.3 (0.4-63.8) | 0.506 |
| 21 days | 20 | 6.9 (1.2-66.6) | 17 | 1.7 (0.3-89.2) | 0.001 |
| **Perinatal factors** | | | | | |
| Sepsis | | | | | |
| 0-48 hours | 44 | 10.7 (0.6-104.1) | 12 | 13.2 (1.1-39.3) | 0.510 |
| 72 hours | 38 | 17.2 (1.3-63.8) | 12 | 10.3 (2.8-64.5) | 0.481 |
| 21 days | 26 | 5.8 (0.3-89.2) | 10 | 3.5 (0.6-44.5) | 0.437 |
| Respiratory distress | | | | | |
| 0-48 hours | 55 | 11.2 (0.6-104.1) | 4 | 5.7 (0.5-10.4) | 0.110 |
| 72 hours | 49 | 15.3 (1.3-63.8) | 4 | 4.8 (0.4-27.1) | 0.148 |
| 21 days | 35 | 4.6 (0.3-89.2) | 4 | 2.4 (0.3-66.1) | 0.431 |
| Invasive mechanical ventilation | | | | | |
| 0-48 hours | 32 | 11.3 (0.6-55.8) | 27 | 7.4 (0.5-104.1) | 0.976 |
| 72 hours | 28 | 17.5 (1.3-46.3) | 25 | 12.7 (0.4-63.8) | 0.873 |
| 21 days | 18 | 4.4 (0.3-89.2) | 21 | 3.9 (0.3-66.1) | 0.382 |
| Non-invasive ventilation | | | | | |
| 0-48 hours | 43 | 11.1 (0.6-104.1) | 16 | 9.6 (0.5-18.1) | 0.331 |
| 72 hours | 38 | 14.8 (1.3-63.8) | 15 | 11.9 (0.4-39.5) | 0.540 |
| 21 days | 30 | 4.2 (0.3-66.6) | 9 | 4.2 (0.3-89.2) | 0.909 |
| Vasoactive medication | | | | | |
| 0-48 hours | 23 | 11.4 (0.6-74.3) | 36 | 7.6 (0.5-104.1) | 0.263 |
| 72 hours | 19 | 15.2 (3.8-46.3) | 34 | 13.0 (0.4-63.8) | 0.553 |
| 21 days | 7 | 9.7 (1.2-89.16) | 32 | 3.8 (0.3-66.6) | 0.154 |
| Nephrotoxic medication | | | | | |
| 0-48 hours | 56 | 11.2 (0.6-104.1) | 3 | 4.4 (0.5-7.0) | 0.067 |
| 72 hours | 50 | 15.9 (1.3-63.8) | 3 | 1.0 (0.4-8.6) | 0.026 |
| 21 days | 36 | 4.6 (0.3-89.2) | 3 | 1.0 (0.3-3.9) | 0.073 |
| Diuretics | | | | | |
| 0-48 hours | 6 | 6.0 (0.6-39.3) | 53 | 11.2 (0.5-104.1) | 0.201 |
| 72 hours | 6 | 22.1 (4.6-39.6) | 47 | 13.2 (0.4-63.8) | 0.801 |
| 21 days | 3 | 31.0 (17.3-35.5) | 36 | 3.8 (0.3-89.2) | 0.073 |

[a]ng/mg, [b]Mann-Whitney

(range 0.72-3.48) g/kg BW protein administered per day. These protein intake levels were not significantly correlated with the uNGAL/Cr ratio. However, the pattern in **Figure 3A** shows that the uNGAL/Cr ratio increased proportionally with the increase in protein intake although not statistically significant (r=0.03, P=0.80). During the first 48 hours, VPN experience physiological and hemodynamic stress in

**Table 3**. Pearson's correlation of uNGAL/Cr and selected variables

| Timepoint | Gestational age | Birth weight | APGAR 1 min | APGAR 5 min |
|---|---|---|---|---|
| 0-48 hours | -0.185 (0.160) | -0.192 (0.146) | 0.228 (0.083) | 0.214 (0.104) |
| 72 hours | -0.167 (0.233) | -0.320 (0.019) | 0.158 (0.239) | 0.215 (0.122) |
| 21 days | -0.134 (0.417) | -0.282 (0.082) | -0.209 (0.201) | -0.142 (0.388) |

Data presented as r (P value)

their new extra-uterine environment, which impacts uNGAL/Cr ratio. Furthermore, tubular immaturity due to immature resorptive function during the early neonatal period may result in an increase of the uNGAL/Cr ratio along with an increase in protein intake. In contrast to post-natal age 0-48 hours, at post-natal age 72 hours and 21 days, the higher the protein intake, the lower the uNGAL/Cr ratio (**Figure 3B and 3C**). The tubular maturation process that occurs with age contributes to improved tubular resorptive function of uNGAL.

Previous studies showed no impairment of tubules and glomeruli in very preterm infants with protein intake levels up to 3.6 g/kg/day at the age of 14 days,16 similar to our study whose participants received same amount of protein intake within the time frame of 72 hours to 21 days. When comparing protein intake between low (<3 g/kg/day) and high (>3 g/kg/day) groups, we also found no difference in uNGAL/Cr ratio (**Figure 2**). The aforementioned study measured serum urea, creatinine, cystatin C and urinary β2 microglobulin as biomarkers for glomerular and tubular injury. The neonatal protein intake concentrations in our study at post-natal ages 0-48 hours, 72 hours, and 21 days were in the range of their study, which was 3 to 3.6 g/kg/day.[16] Another study reported a decrease in eGFR, sodium clearance, and osmolality with the administration of breastmilk and supplemental protein up to 7 g.[15] We also found that the median protein intake received by participants at post-natal age 0-48 hours [2.09 (range 0.72- 3.48) g/kg/day], 72 hours [3.12 (range 0.87- 4) g/kg/day], and 21 days [3.44 (range 2-5.18) g/kg/day] were lower than our NICU guidelines. Even though the protein intake was lower than expected, the weight-to-age z-score (WAZ) at 21 days post-natal age according to the IPNG curve showed that 32 of 39 VPN had an increase in body weight compared to their birth weight. This finding supports a previous systematic review which found that 3 g/kg/day protein administration is needed for making up growth in VPN.[18]

The traditional AKI definition by serum creatinine and urine production criteria detects AKI only after at least 50% of nephron injury occurs. Creatinine is influenced by maternal serum creatinine level, muscle mass, hydration state, and sex.[24] During AKI, tubular injury occurs before the increase in serum creatinine. Biomarkers were developed to detect early tubular injury so that AKI can be detected and managed earlier. These biomarkers can be detected in blood and/or urine. Other urinary biomarkers such as urine β2 microglobulin, KIM-1, and urine LFABP were studied in preterm neonates for early detection of kidney injury. These biomarkers were measured in urine specimens, the collection of which were less invasive than blood draws for VPN.[11] Serum creatinine was measured only when indicated, and not part of this study. Thus, we could not measure the prevalence of AKI in our study.

In maternal conditions such as infection, anemia, hypertension, and diabetes, placental blood flow usually decreases, which may affect fetal renal blood flow, ultimately causing a hypoperfusion of kidney cells and kidney injury. Moreover, maternal infection commonly leads to early onset sepsis, which increases the risk of AKI in neonates. Our study showed that maternal infection and maternal anemia were associated with higher uNGAL/Cr ratio (**Table 2**).

Participants who were exposed to nephrotoxic antibiotics had higher uNGAL/Cr ratio. Nephrotoxic medication given to VPN in our study included gentamicin and/or amikacin. Gentamicin and amikacin cause tubular injury by damaging proximal tubule segments S1 and S2, whilst NGAL is mostly produced as a result of damage of the proximal tubule.[25] The Baby NINJA Study reported positive associations between the number of nephrotoxic agents administered in the NICU and increased risk of AKI in very preterm infants.[20] In our study, 10.2%

of VPN received diuretic (furosemide) treatment, whereas a previous study across 46 US children's hospitals reported 17.9% use of diuretics in preterm neonates.[26] Participants who received diuretics had higher median uNGAL/Cr ratio at 72 hours and 21 days (**Table 2**). Diuretics are usually given as a treatment option for AKI in neonates due to the lack of appropriately-sized kidney support therapy devices, especially for those with smaller body weight, therefore, diuretics are the mainstay therapy.[26]

We found that the uNGAL/Cr ratio was inversely related to gestational age and birth weight in VPN. As reported in a previous study, gestational age was the only parameter affecting uNGAL/Cr ratio in a mixed model regression analysis compared to other parameters, such as sex, race, prevalence of AKI, and insertion of an umbilical artery catheter.[27] Participants with higher gestational ages showed lower uNGAL/Cr ratio at all timepoints (**Table 3**). In VPN, gestational age is a critical factor that affects the quantity of functional nephrons, the ability of the nephrons to filter, and the maturation of renal tubules.[28] Similarly, the uNGAL/Cr ratio has been negatively correlated with birthweight,[27,29] as seen in **Table 3**. A significant correlation at 72 hours between a decrease in the uNGAL/Cr ratio and an increase in birthweight was observed in our study (P=0.019). The level of uNGAL/Cr ratio decreased by 13.4% with a 100 g increase in birthweight (95%CI -16.6 to -10.1), and decreased by 11.3% with a 1 week increase in gestational age (95%CI -15.3 to -7.1). Nonetheless, uNGAL/Cr ratio decreased by 4.4% with a 1-day increase in post-natal age (95%CI -5.6 to -3.2).[30]

The uNGAL levels were significantly elevated in asphyxiated neonates.[31] Neonatal asphyxia is the leading cause of kidney ischemia and hypoxia. Decreased kidney blood flow is a major cause of overly expressed NGAL in renal tubules. One Indonesian study showed significantly higher median urinary NGAL levels in the asphyxiated group [506.7 (60.0 - 651.7) ng/mL] than in the non-asphyxiated group [6.7 (0.1 - 53.0) ng/mL]; (P<0.001).[31] One of the criteria of asphyxia in the aforementioned study was Apgar score of 0 to 3 for longer than 5 minutes. In our study, we did not have any participants with asphyxia, as median APGAR score was 6 (range 2-8) at 1 minute and 8 (range 5-10) at 5 minutes. Therefore, we did not find any correlation between APGAR score and uNGAL/Cr ratio.

The main strength of our study was the adequate sample size used to show statistical insignificant correlation between protein intake and uNGAL/Cr level. We also included only VPN with appropriate gestational ages, which presumably excluded a possible confounding effect of low birth weight on tubular injury. Urine specimens were not collected on a daily basis, therefore, variation in the post-natal period could have been missed. We were also unable to control exposure to maternal and perinatal factors during our study observation. However, we included those factors in our analysis. Since our hospital is a tertiary hospital, most VPN were referral cases with no prior records of antenatal ultrasound. However, any clinical issues regarding congenital anomaly of the kidney and urinary tract found after delivery would have been referred to a pediatric nephrologist as per protocol. None of the participants received pediatric nephrologist consultations.

In conclusion, our study shows the amount of protein intake according to current nutritional guidelines does not correlate with tubular injury in VPN, as measured by uNGAL/Cr ratio. Thus, our findings support the current nutritional guidelines for VPN, which recommend administering high protein levels to promote growth. Furthermore, maternal infection, maternal anemia, lower birth weight, and nephrotoxic medication are associated with higher uNGAL/Cr levels in VPN.

## Conflict of interest

None declared.

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