ASSESSMENT OF RENAL FUNCTIONAL MATURATION AND INJURY IN PRETERM NEONATES
DURING THE FIRST MONTH OF LIFE

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Running Head: Renal function and injury in preterm neonates

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Worldwide, approximately ten percent of neonates are born preterm. The majority of preterm neonates are born when the kidneys are still developing; therefore, during the early postnatal period renal function is likely reflective of renal immaturity and/or injury. This study evaluated glomerular and tubular function, and urinary neutrophil gelatinase associated lipocalin (NGAL; a marker of renal injury) in preterm neonates during the first month of life. Preterm and term infants were recruited from Monash Newborn (neonatal intensive care unit at Monash Medical Centre) and Jesse McPherson Private Hospital, respectively. Infants were grouped according to gestational age at birth: ≤ 28 weeks (n=33), 29 – 31 weeks (n=44), 32 – 36 weeks (n=32) and term (≥ 37 weeks (n=22)). Measures of glomerular and tubular function were assessed on postnatal days 3-7, 14, 21 and 28. Glomerular and tubular function was significantly affected by gestational age at birth, as well as postnatal age. By postnatal day 28, creatinine clearance remained significantly lower among preterm neonates compared to term infants; however, sodium excretion was not significantly different. Pathological proteinuria and high urinary NGAL levels were observed in a number of neonates which may be indicative of renal injury; however, there was no correlation between the two markers. Findings suggest that neonatal renal function is predominantly influenced by renal maturity and there was high capacity for postnatal tubular maturation among preterm neonates. There is insufficient evidence to suggest that urinary NGAL is a useful marker of renal injury in the preterm neonate.
INTRODUCTION

Renal function in the preterm neonate is affected by renal immaturity and potential injury during the early postnatal period. At the time when the majority of preterm infants are born, renal development is still ongoing (48) and renal function is accordingly immature (15). Preterm neonates have been shown to have a low glomerular filtration rate (GFR) compared to term neonates, and the tubules excrete high amounts of sodium (2). Furthermore, compared to babies born at term, preterm neonates may demonstrate a slower progression in renal functional maturation after birth (5, 14). Creatinine clearance ($C_{Cr}$) has been shown to be positively correlated with both gestational age and postnatal age (2, 5, 7-9, 12, 14, 21, 39, 47, 51, 56), while the fractional excretion of sodium ($F_{ENa}$) has been shown to be inversely correlated with gestational age (12) and postnatal age (2, 12, 13, 39, 47).

Only a few studies to date have investigated the occurrence of proteinuria following preterm birth. Very low concentrations of both high molecular weight (HMW) and low molecular weight (LMW) proteins are normally present in the urine, due to the function of the glomerular filtration barrier and the reuptake of filtered proteins in the proximal tubule (28, 32). A limited number of previous studies have demonstrated a high variation in urine albumin levels between individual preterm neonates (7, 10), with the highest levels exhibited by those with a low gestational age at birth and those that are clinically unstable (3, 7, 10, 11, 52). Urinary levels of $\beta_2$-microglobulin have also been shown to be significantly greater in the preterm infant compared to term-born infants throughout the first month of life (2, 52, 53), and they decrease with increasing gestational and postnatal age (50). Proteinuria is known to be an important indicator of renal injury; however, to date it remains unclear whether the increased urinary protein levels reported in preterm neonates are associated with renal immaturity and/or acute renal injury.

The preterm kidney is highly susceptible to injury in the neonatal period; acute kidney injury (AKI) is reported to occur in 8% to 24% of preterm neonates admitted to the neonatal intensive care unit (19, 44) and is primarily pre-renal in origin (6, 44). In a large study of preterm infants born in the USA and Puerto Rico, Walker et al. (55) examined the medical records of 66,526
neonates (born at ≤ 30 weeks gestation); 4% of the neonates were diagnosed with renal
dysfunction and/or renal failure. The predominant risk factors for impaired renal function were
low gestational age and low birth weight. Further risk factors included postnatal medication
administration (vasopressor, indomethacin, and antibiotics), postnatal illness (intraventricular
haemorrhage, a patent ductus arteriosus, necrotising enterocolitis, culture positive sepsis) and
also the use of high frequency ventilation, male gender and non-white race. Importantly,
mortality rates were significantly higher in neonates with the diagnosis of renal dysfunction
and/or renal failure (55). In addition, renal injury in the preterm neonate may be an
antecedent to chronic renal disease (1). Therefore, the early diagnosis of AKI is paramount so
that conservative management of AKI can be initiated in time to potentially prevent these long-
term consequences. In this regard, urinary neutrophil gelatinase associated lipocalin (NGAL)
has recently been investigated as a potential biomarker of AKI in preterm neonates, with
studies showing that NGAL levels strongly correlated with both gestational and postnatal age
(20, 23), and are highest in neonates that are critically ill (23, 33).

In this Australian-based study, we have examined renal function in preterm infants admitted to
the neonatal intensive care unit at the Monash Medical Centre (a large tertiary level hospital,
located in Melbourne, Australia); the current rate of preterm birth in the Australian population
is 8.2% (24). The aims of the study were to: 1) assess postnatal renal function from day 3 to day
28 in extremely preterm (≤ 28 weeks of gestation), very preterm (29 – 31 weeks of gestation),
and moderately preterm (32 – 36 weeks of gestation) neonates compared to term controls (37-
42 weeks gestation) by examining glomerular (C\text{Cr}, and urine albumin) and tubular (FE\text{Na} and
urine β2-microglobulin) function; and 2) to determine whether urinary NGAL is a useful marker
of renal injury in preterm neonates.
MATERIALS & METHODS

Ethics statement

Ethics approval for this study was obtained from the Southern Health Human Research Ethics Committee and the Monash University Standing Committee on Ethics in Research Involving Humans. Written informed parental consent was obtained for all participants in the study.

Study population

Preterm neonates (<37 weeks of gestation) admitted to the neonatal intensive care unit at Monash Medical Centre and term infants (37-42 weeks gestation) born at Jessie MacPherson Private Hospital (Clayton, Victoria, Australia) without any congenital abnormalities, were eligible for the study. Between April 2008 and October 2011, 143 neonates were recruited into the study. Four neonates were excluded following the early withdrawal of parental consent. Eight preterm neonates were further excluded following transfer to other hospitals prior to postnatal day 7. Two extremely preterm neonates died before the study was completed. The remaining 129 neonates were stratified into four groups according to gestational age: Group A (≤ 28 weeks gestation; n=33), Group B (29 – 31 weeks gestation; n=44), Group C (32 – 36 weeks gestation; n=30), and Group D (≥ 37 weeks gestation; n=22).

Urine collection procedure

Sanitary pads (Kotex; Kimberly-Clark, NSW, Australia) were placed within nappies in order to collect urine samples, a method that has been previously validated (18, 45). A nappy liner (Johnson’s Baby Nappy Liners; Johnson & Johnson Pacific, NSW, Australia) was also placed inside the nappy to filter out any faeces. In order to estimate urine flow rate, all nappies inclusive of the pad and nappy liner were weighed before and after use, and the time each nappy was put on the baby was recorded (38). In the case of missed voids or a heavily soiled nappy, a value of average urine output for the relevant time period (calculated from all other nappies within that 24 hour period) was substituted for the missing value. Nappies were changed at the discretion of nursing staff and/or parents (ranging from every 8 hours in the extremely preterm neonates, to less than 2 hours in the term neonates), and collected in a
sealed plastic container. At least twice per day, nappies were collected from the nursery and the urine was extracted by compressing the sanitary pad using a hydraulic press (18, 45). All urine collected from the nappies over a 24 hour period was pooled before analysis.

It has been shown that urine collected from disposable cotton pads and/or cotton wool does not affect the urinary constituent of sodium, potassium or creatinine (38) and has been used previously in the analysis of urinary NGAL (23). Previous research has shown that protein can get bound within cotton material (43). Therefore, spot urine samples collected using urine collection bags were utilised to determine urine total protein, albumin and β2-microglobulin levels.

In preterm neonates, 24 hour urine collection from nappies began at 72 hours after birth (day 3) and continued until postnatal day 7. In addition, 24 hour urine was collected on days 14, 21 and 28 of life. Spot urine samples (1-2 ml) were obtained on days 7, 14, 21 and 28 of life. In term neonates, 24 hour urine collection commenced 48 hours after birth (day 2) and continued until the infant was discharged from hospital (approximately day 4 of life). Additionally, urine was collected for a 24 hour period on day 28 of life. For those infants who had been discharged, nappies were delivered and collected from the infant’s home (Group A n=0, Group B n=0, Group C n=7, Group D n=11). Spot urine samples were obtained from the term neonates on day 3 of life. Pooled 24 hour urine samples were frozen at -20°C until analysis. Spot urine samples were sent for analysis of urinary protein levels immediately after collection.

For a number of the infants, the urine collections and analyses on days 14-28 were not performed primarily due to the transfer of infants to private hospitals, or discharge from hospital; Group A (n=1/33 (3.0%), Group B (n=8/44 (18.2%)), Group C (n=11/30 (33.3%), and Group D (n=11/22 (50.0%)).

Assessment of renal function

**Urinary and plasma sodium and creatinine**

All urine analyses were performed by the Southern Health Pathology Department (Southern Cross Pathology; Clayton, Victoria, Australia). Pooled 24 hour urine samples were analysed for
sodium and creatinine levels. Plasma creatinine and plasma sodium levels were recorded from blood tests that were undertaken as part of the routine care of preterm neonates, and data were extracted from the medical records. In term neonates, heel-prick blood samples were obtained for analysis at the time of the routine newborn screening test (at approximately 48 hours of life). In cases where a blood test was not available in term infants on postnatal day 28 (Group D, n=11), average levels of plasma sodium (140 mmol/L) and plasma creatinine (40 µmol/L) that are within the expected range for term infants were used in the calculation of \( C_{Cr} \) and \( FE_{Na} \).

### Calculation of \( C_{Cr} \) and \( FE_{Na} \)

The estimation of glomerular filtration rate (GFR) using \( C_{Cr} \) is generally considered to be a more accurate measure of glomerular function than serum creatinine alone. Although it is to be noted that the potential for active tubular secretion of creatinine, as well as differences in muscle mass between neonates, may affect the accuracy of the results (46).

\( C_{Cr} \) was calculated using the following formula:

\[
C_{Cr} \text{ (ml/min/BSA)} = \frac{UCr / PCr \times \text{Urine Flow Rate}} {\text{BSA}}
\]

Where \( UCr = \) urinary creatinine (µmol/L); \( \text{Urine Flow Rate} = \) ml/min (calculated for each 24 hr period); \( PCr = \) plasma creatinine (µmol/L); \( \text{BSA} = \) body surface area (m²).

Body surface area (BSA) was calculated using the following formula derived by Haycock et al. (17):

\[
\text{BSA (m}^2) = 0.024265 \times \text{body weight (kg)}^{0.5378} \times \text{body length (cm)}^{0.3964}
\]

The fractional excretion of sodium (\( FE_{Na} \)) was calculated using the following formula:

\[
FE_{Na} \text{ (%) = } \frac{UNa \times PCr}{PNa \times UCr} \times 100
\]

Where \( UNa = \) urinary sodium (mmol/L); \( PNa = \) plasma sodium (mmol/L); \( PCr = \) plasma creatinine (µmol/L); \( UCr = \) urinary creatinine (µmol/L).
Urinary total protein, albumin and β2-microglobulin

Urinary total protein (UTP), urinary albumin and urinary β2-microglobulin (β2-M) were measured in spot urine samples using nephelometric technology on a Beckman immunochemistry system, with reagents and calibrators supplied by Beckman Diagnostics (urine total protein and urine albumin; Beckman Diagnostics; Sydney, Australia) and DakoCytomation (β2-microglobulin; DakoCytomation; Glostrup, Denmark). In instances where UTP was greater than 500 mg/L, this was defined as pathological proteinuria and urinary albumin levels were not determined. All urine protein levels were expressed as a ratio to urine creatinine concentrations.

It is to be noted that missing data exists for the urinary protein results due to difficulties in obtaining clean spot urine samples from a number of the infants (urine collection bags could not be placed on some extremely preterm neonates due to their delicate skin and/or parents not providing consent). Overall, 46.2% of the total number of requested spot urine samples were obtained. At least one spot urine sample was obtained for the majority of neonates: Group A (91%), Group B (93%), Group C (87%), and Group D (27%).

Urinary NGAL

In a subset of the study participants (61.2%), pooled 24 hour urine samples from one or more postnatal time points were analysed for urinary NGAL levels. Urinary NGAL analysis was performed using a sandwich ELISA in microwells coated with a monoclonal antibody against human NGAL (NGAL ELISA Kit, BioPorto Diagnostics A/S; Gentofte, Denmark); the upper limit of the test was 500 ng/ml. In accordance with previous studies, urinary NGAL levels were expressed as ng/ml, and were not corrected for urine creatinine concentrations (20, 23, 33).

Impaired renal function

Impaired renal function (low urine output, low creatinine clearance, high serum creatinine, high fractional excretion of sodium, high urine total protein and high urinary NGAL) was defined as values that differed more than 2 standard deviations from the mean at any time point from postnatal day 3 through to postnatal day 28. The mean and standard deviation was calculated from either absolute values (SCr) or natural log (urine output, serum Cr, FE_{Na}, UTP, NGAL) or
square-root ($C_r$) transformed values (transformations were performed in order to ensure the normality of data), inclusive of all preterm neonates (Groups A, B and C). In order to assess the relationship between the six measures of renal dysfunction and whether any preterm neonate exhibited multiple measures of renal impairment, the total number of measures of renal dysfunction for each neonate was also examined.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism v5.04 for Windows (CA, U.S.A.) and Intercooled Stata v8.0 for Windows (TX, USA). Data are presented as the mean ± standard error of the mean (SEM). Statistical significance was accepted at the level of $p<0.05$.

Birth characteristics (gestational age, birth weight, length, head circumference) were compared among groups using a one-way analysis of variance (ANOVA), followed by a Bonferroni post-hoc test. To determine differences in categorical variables among groups (such as sex, disease outcomes, and medication administration), a Fisher’s exact test was performed.

Urine output, $C_r$ and the $F_E_{Na}$ in preterm neonates (Groups A, B and C) were analysed using a two-way ANOVA with repeated measures in order to assess renal functional maturation with increasing postnatal age. At postnatal day 3 and day 28, urine output, $C_r$ and the $F_E_{Na}$ (in all four groups) was analysed using a two-way ANOVA, followed by a Bonferroni post-hoc test to determine differences between individual groups at each time point; this analysis enabled comparison between preterm and term infants at the start- and end-points of the study. Urine protein (UTP, albumin, and $\beta_2$-M) to creatinine ratios and urine NGAL levels were also analysed using a two-way ANOVA, followed by a Bonferroni post-hoc test. The factors assessed in all of these analyses were gestational age ($p_{GA}$), postnatal age ($p_{PA}$) and their interaction ($p_{GA} \times p_{PA}$).

Linear regression analyses (followed by an analysis of covariance (ANCOVA)) were used to compare the rate of change in $C_r$ and $F_E_{Na}$ from postnatal day 3 to day 28, and also to determine whether urinary NGAL levels correlated with any other indices of renal function. Additionally, urinary NGAL levels in neonates exhibiting pathological proteinuria (UTP $\geq$ 500 mg/L) were compared to age and sex-matched neonates (controls) with lower urine total
protein levels (UTP < 480 mg/L, and less than half of the UTP level of the matched neonate with pathological proteinuria) at the corresponding postnatal time point. This analysis was undertaken using a two-way ANOVA with the factors proteinuria (p_P), gestational age (p_GA) and their interaction (p_P x G_A). Additionally, in these two groups, urinary NGAL levels were assessed at time points prior to (7 days prior if pathological proteinuria observed at day 14, 21 or 28; 3 days prior if observed at day 7), and at the time of proteinuria onset. This analysis was undertaken using a two-way ANOVA with the factors proteinuria (p_P), time point of assessment (p_T) and their interaction (p_P x T).
RESULTS

Pregnancy and neonatal birth characteristics

Reasons for preterm delivery included onset of spontaneous preterm labour (34.0%), premature pre-labour rupture of membranes (30.1%), placenta praevia/abruption (15.5%) and clinical indication due to maternal and/or fetal health risks such as preeclampsia and suspected fetal compromise (20.4%). There were a significantly higher proportion of births attributed to spontaneous preterm labour in Group A (extremely preterm) neonates compared to both Group B and Group C neonates. The majority of preterm neonates (> 65%) were born via caesarean delivery, compared to 27% among term infants (Table 1). At least 70% of the mothers of preterm neonates received antenatal steroids prior to delivery; 94% of those born extremely preterm (Group A) received antenatal steroids.

Body weight, length and head circumference at birth all significantly increased along with increasing gestational age (Table 1). There was no significant difference in the gender balance between gestational age groups. Groups A and C had the greatest number of small-for-gestational age (SGA) neonates; there was a similar number of multiple births in each of the preterm groups whereas all term-born neonates in Group D were singletons. Of the preterm groups, there was no significant difference in birth weight between the singletons and multiples (Group A: singleton 818.6 ± 36.8 g, multiple 790.9 ± 35.8 g; Group B: singleton 1429 ± 47.9 g, multiple 1468 ± 65.0 g; Group C: singleton 1767 ± 111.4 g, multiple 1778 ± 93.4 g). The majority of neonates that had a low Apgar score (≤7) at 5 minutes were in Group A (Table 1).

Postnatal neonatal complications and medications administered

Since the majority of preterm neonates required mechanical ventilation after birth, respiratory distress syndrome was very common in these groups (Table 2). The occurrence of culture-positive sepsis and a patent ductus arteriosus was significantly greater in Group A compared to those infants in older gestational age groups. Only neonates within Group A and Group B were diagnosed with intraventricular haemorrhage. Among preterm neonates, the most commonly administered medications were a routine regimen of antibiotics. The majority of neonates that
received additional antibiotics were in the extremely preterm group (Group A). In general, the administration of these drugs was significantly greater among neonates in Group A. Term infants (Group D) did not receive any medications over the course of the study.

Renal function

Urine output

Neither gestational age at birth nor postnatal age affected urine output in preterm neonates from postnatal days 3 to 7 (Figure 1A). On postnatal days 14 to 28, however, urine output was significantly greater in neonates with increased gestational age at birth (Figure 1B). Urine output was significantly lower in term neonates compared to preterm neonates at postnatal day 3, however there was no difference between groups at postnatal day 28 (Figure 1C). Oliguria (urine output < 1 ml/kg/h) was observed in 10 term infants (postnatal day 3) and 1 preterm neonate (postnatal day 5).

C<sub>cr</sub>

During the first week and month of life, C<sub>cr</sub> was positively associated with both gestational age at birth and postnatal age (Figure 1D and 1E). Of the preterm neonates, Groups B and C had the highest C<sub>cr</sub> throughout the study period. On postnatal day 28, C<sub>cr</sub> levels were not significantly different among the preterm groups, however, term neonates had significantly higher C<sub>cr</sub> compared to preterm neonates (Figure 1F). Linear regression analyses showed that there was no significant difference in the rate of change in C<sub>cr</sub> from postnatal day 3 to postnatal day 28 among all groups of neonates (data not shown).

FE<sub>Na</sub>

The FE<sub>Na</sub> was inversely associated with gestational age at birth during the first week and month of life (Figures 1G and 1H). The highest levels of sodium excretion were observed in Group A throughout the study period. On postnatal day 3 (Figure 1I) FE<sub>Na</sub> was significantly lower in term neonates (Group D) than all other groups. By postnatal day 28, however, there was no significant difference in FE<sub>Na</sub> among groups. Linear regression analysis showed that the rate of
change of $\text{FE}_\text{Na}$ from postnatal day 3 to day 28 was significantly greater in Group B neonates compared to Group D ($p<0.0001$), and was also significantly greater in Group A neonates compared to all other groups ($p \leq 0.02$; data not shown).

**UTP, albumin, and $\beta2$-M**

UTP, albumin and $\beta2$-M levels in preterm neonates (corrected for urine creatinine) were all inversely associated with gestational age at birth (Figure 2). There was no change in urine protein levels with increasing postnatal age. There was wide intra-group variability in urine protein levels; within Group A for example, UTP:Cr levels ranged from 92.2 mg/mmol to 759.3 mg/mmol at postnatal day 28 (Figure 2A). In term infants UTP:Cr (mean: 219.3 ± 139.9 mg/mmol) and albumin:Cr (mean: 19.9 ± 9.1 mg/mmol) levels measured on postnatal day 3 were similar to the levels found among Group B neonates at postnatal day 7; $\beta2$-M:Cr levels (mean: 0.8 ± 0.2 mg/mmol), however, were negligible. Pathological proteinuria (UTP $\geq 500$ mg/L) was observed in 12 (9.3%) neonates, at one or more postnatal time points, with the majority of these in Group A (7/12), followed by Group B (2/12), Group C (1/12) and Group D (2/12).

**Urinary NGAL**

There was a significant effect of gestational age on urinary NGAL, with the lowest NGAL levels observed at postnatal day 28 in Group D term neonates (Figure 3A). Nine preterm neonates (7.0%) had levels of urinary NGAL $> 390$ ng/ml ($> 2$ SD from the mean) at one or more postnatal time points with the majority (7/9) occurring on postnatal day 28.

There was a significant positive linear correlation between urinary NGAL levels and UTP:Cr (Figure 3B), however, urinary NGAL levels were not correlated with serum creatinine, $C_{\text{Cr}}$, $\text{FE}_\text{Na}$, urine albumin or $\beta$-2 microglobulin levels (data not shown). Urinary NGAL levels in the subset of preterm neonates who exhibited pathological proteinuria (UTP $\geq 500$ mg/L) were not different to the NGAL levels of age and sex-matched controls with low protein excretion at corresponding postnatal time points (Figure 3C). In these two groups, with the exception of a few neonates, urinary NGAL levels were not predictive of proteinuria (Figure 3D); urinary NGAL
levels were not significantly increased at the time points prior to, or at the onset of pathological proteinuria).

**Impaired Renal Function**

Overall, approximately 14% of preterm neonates from each gestational age group were classified as having low urine output (Figure 4A). Twenty-five percent of neonates in Group A, 9.5% in Group B, and 7.1% in Group C had high serum creatinine levels (> 100.6 µmol/L); only one neonate (Group B), however, was categorised as having low CCr (Figures 4B and 4C). High FE$_{Na}$ were predominantly observed in Group A neonates, with 3 preterm neonates from Group A and B exhibiting hyponatraemia (serum sodium levels < 130 mmol/L) during the study period (Figure 4D). High UTP levels were only observed in Group B and Group C neonates, whereas high urinary NGAL levels were observed in a small percentage of neonates in each gestational age group (Figures 4E and 4F).

The relationship between the six different measures of renal dysfunction (described in Figure 4), and whether any preterm neonates had multiple measures of renal impairment, was also examined. Twelve (11.2%) preterm neonates had more than two measures of renal impairment. As shown in Table 3, the most common combination of renal dysfunction measures exhibited during the first month of life was high serum creatinine and high fractional excretion of sodium (observed in 7 of 12 neonates).
The findings of this study demonstrate that renal function in preterm neonates, during the first month of life, is significantly affected by gestational age at birth and postnatal age. Together, these results suggest that neonatal renal function is predominantly influenced by renal structural maturity. By postnatal day 28, $C_{Cr}$ was significantly lower among preterm neonates compared to term infants, however, differences in $FE_{Na}$ were not observed which is suggestive of a high capacity for postnatal tubular maturation. Both urinary protein and NGAL levels were inversely associated with gestational age at birth which suggests that they are markers of renal immaturity. Of concern, pathological proteinuria was observed in 12 preterm neonates; among those neonates, urinary NGAL levels were not elevated which has two potential implications: 1) that the cause of the pathological proteinuria is not due to acute kidney injury (as NGAL has previously been shown to be a marker of AKI) or 2) if the pathological proteinuria observed is due to renal injury, urinary NGAL is not a useful marker or predictor of renal injury in this population. The findings from this study provide normative values for a number of renal functional parameters in preterm infants. The presence of renal dysfunction in a high proportion of babies highlights the importance of conducting large-scale studies among populations of preterm neonates and the close monitoring of renal function in preterm infants in the neonatal intensive care unit (NICU).

**Maturation of renal function in the early postnatal period**

In this study, urine output was not significantly different during the first week of life among preterm neonates. This finding was expected, as within the NICU fluid intake is strictly maintained according to neonatal body weight, and from approximately postnatal day 4 urine output is known to be predominantly influenced by fluid intake (26, 27). From days 14-28, a significantly higher urine output was observed among neonates born at older gestational ages, which likely relates to the change in feeding regime. All term infants were exclusively breast-fed in the first week after birth; hence their observed low urine output is likely due to low maternal milk production within the first few days after birth. Since this was a non-invasive study, urine output was estimated using nappy weights; it is important to note that this method has its
limitations since estimates based on averages had to be used in occasional instances of missed
voids and soiled nappies. The 24 hour urine collections conducted at home were also highly
reliant on the record-keeping of parents, and this non-invasive technique was therefore prone
to error. Furthermore, the number of infants assessed after postnatal day 7 was reduced due
to loss to follow-up; therefore the robustness of data was diminished for all analyses conducted
on postnatal days 14-28. Consistent with previous studies, as gestational age at birth and
postnatal age increased, C\text{Cr} increased (2, 5, 7-9, 12, 14, 21, 39, 47, 51, 56) and FENa decreased
(2, 12, 13, 39, 47). Although neonates born very or extremely preterm commence with a low
GFR and high FENa, by postnatal day 28 both C\text{Cr} and FENa were not significantly different
between the preterm gestational age groups which is suggestive of a high capacity for postnatal
glomerular and tubular maturation among those born very or extremely preterm. Interestingly,
the rate of change in \text{C}Cr over the first month of life was very similar between all groups of
neonates, and in contrast to previous studies (5, 14), a slower postnatal increase in \text{C}Cr in
preterm neonates compared to term infants was not observed. In extremely preterm neonates,
there was a significantly greater rate of change in FENa from postnatal day 3 to day 28 compared
to all other groups suggestive of more accelerated tubular maturation. In support of this idea,
at postnatal day 28 C\text{Cr} remained significantly lower among preterm neonates compared to
term infants whereas there was no significant difference in FENa. Overall, these results suggest
that renal functional capacity increases with renal structural maturity; potentially, through
increased filtration surface area (increased number of nephrons in those neonates with ongoing
postnatal nephrogenesis\textsuperscript{(48)} and/or increased capillary growth) (31, 48), the maturation of
tubular cells (40) as well as the substantial changes in renal blood flow and renal vascular
resistance that occur after birth (54).

In accordance with previous studies (2, 3, 7, 10, 11, 52, 53), we observed that urine albumin,
\text{\beta}2-M and UTP levels were inversely associated with gestational age at birth. There was no
change, however, in urine protein levels with increasing postnatal age. Proteinuria in the
preterm neonate may reflect either structural immaturity or injury to the glomerular filtration
barrier (allowing for the passage of albumin into the filtrate), and/or impaired uptake of filtered
protein in the proximal tubule. Unlike the significant reduction in sodium excretion with
increasing maturity, the capacity for protein reabsorption remained low postnatally which possibly reflects a much slower maturation of renal protein handling compared to sodium handling. Alternatively, these findings may be indicative of glomerular or tubular injury in the preterm neonate; proximal tubule cell injury (such as occurs following oxidative stress in the preterm neonate (36)), and/or an overload of filtered protein are possible causes of impaired tubular protein uptake.

Evidence of renal dysfunction in preterm neonates

In this study, renal impairment was defined as measures of renal function that were greater than 2 standard deviations from the mean. There is a current lack of definition in the literature as to what constitutes AKI in the preterm neonate; if the general definition of AKI commonly used in adults (RIFLE criteria (4, 30)) was applied in the current study, only 1 preterm neonate (and 10 term neonates presenting with oliguria) clearly met the criteria. Therefore, we adopted a broader definition of dysfunction in the current study in order to give an indication as to the percentage of infants with reduced renal functional capacity (glomerular and tubular), rather than focus on the strict AKI criteria.

In the present study, 25% of neonates in Group A, and less than 10% in Groups B and C, exhibited high serum creatinine levels (> 2 SD from the mean); there was just one preterm neonate (Group B), however, that had low C$_{Cr}$ levels (from day 3-6 of life). A relatively high percentage of preterm neonates, predominantly those born extremely preterm, were observed to have a high percentage of sodium excretion; however, likely due to the administration of sodium supplementation in this population, the majority of preterm neonates maintained adequate levels of serum sodium. It may be speculated that the high sodium excretion in these neonates represents either tubular immaturity or injury. Of the neonates that exhibited more than one measure of renal dysfunction, the majority had both high FENa and high serum creatinine. If tubular function is impaired, this may also have an impact on serum creatinine levels as creatinine (besides predominantly being filtered by the glomeruli) is known to be actively excreted by proximal tubular cells (25, 35), and in the case of preterm neonates, may also be reabsorbed by the immature tubules as has been observed in a neonatal animal model.
Serum creatinine levels are also influenced by extrarenal factors such as muscle mass, and the intake of nitrogen, protein and creatinine which is increased with milk formula and other parenteral nutrition preparations. Each of these factors may have influenced the relatively high proportion of neonates who also exhibited high serum creatinine levels; in contrast, only 1 preterm neonate was found to have low creatinine clearance. In general, creatinine clearance is considered to be a more reliable indicator of glomerular filtration rate than serum creatinine levels alone, especially given the large number of factors that may influence the generation of creatinine as described above. Encouragingly, this finding may indicate that the capacity for glomerular filtration in the preterm kidney in the early neonatal period is quite adequate; however, tubular function is likely impaired.

Pathological proteinuria was observed among twelve neonates in this study (including two term born infants) at one or more postnatal time points. In this regard, the only preterm neonate with low C\textsubscript{Cr} also exhibited pathological proteinuria on postnatal days 14, 21 and 28. Interestingly, the onset of severe proteinuria was often observed at a later stage (9/12 neonates exhibited pathological proteinuria after day 21), which suggests that factors in the postnatal clinical course (rather than renal immaturity) may be the cause of the severe proteinuria. In support of this idea, it was only neonates born at older gestational ages that exhibited high UTP levels (> 2 SD from the mean). Exposure to nephrotoxic medications (including NSAIDs and antibiotics) are known to impair nephrogenesis and cause renal injury, such as podocyte foot process effacement. It is important to note that in regards to all the maternal and fetal factors we examined (including medications) there was no direct corollary with renal impairment; thus indicating that the renal dysfunction is likely to be multifactorial in origin. Future studies in a larger cohort of neonates are required to identify individual factors (such as exposure to medications and growth restriction) which may be contributing to renal dysfunction following preterm birth.

**Urinary NGAL as a marker of acute kidney injury?**

NGAL is excreted by renal proximal tubule cells as a response to AKI; however, NGAL is produced during nephrogenesis, and levels may also be raised in late-onset sepsis.
Although positive findings have been reported among older infants (57), the usefulness of urinary NGAL as a marker of AKI in preterm neonates remains unclear (20, 23, 33, 34). Certainly, consistent with previous studies (20, 23) we found a significant inverse correlation between urinary NGAL and gestational age at birth; these findings may relate to the immaturity of the kidney, and the clinical instability of the younger neonates. Importantly, the results of the current study demonstrated that urinary NGAL levels were not directly correlated with any indicators of renal dysfunction, apart from UTP. Although the correlation between NGAL and UTP was statistically significant, it is to be noted that the coefficient of determination was very low. Furthermore, only two preterm neonates were observed to have concurrently high UTP and NGAL levels; in general, neonates with pathological proteinuria did not exhibit high urinary NGAL levels. There was also no association between NGAL levels at time points before or at the time of proteinuria onset, suggesting that NGAL may not be predictive of renal injury and/or proteinuria.

Conclusions

The findings of this study demonstrate that renal maturity is an important determinant of glomerular and tubular function among preterm neonates. Of particular concern, a number of preterm neonates exhibited severe proteinuria; however, there was no correlation with urinary NGAL levels. Given the immaturity of the preterm kidney it is important to determine what levels of protein in the urine are normal, versus those that are pathological and thus indicative of renal injury in preterm neonates. Furthermore, to identify specific factors in the postnatal clinical care of the preterm neonate which may be leading to the high urinary protein excretion. The results from this study have been important in working towards the development of a normal range of urinary protein levels in preterm neonates, but our findings are limited by the relatively small sample size and the large inter- and intra-group variability observed. The consequences of proteinuria in the neonatal period are unknown; however, the potential for progressive renal injury and long-term renal dysfunction suggest the need for regular assessments of renal function in subjects that are born preterm.
acknowledgements

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disclosures

None

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REFERENCES


Figure 1: Urine output (A-C), creatinine clearance (D-F), and the fractional excretion of sodium (G-I) in neonates born at ≤ 28 weeks gestation (Group A), 29-31 weeks gestation (Group B), 32-36 weeks gestation (Group C) and ≥ 37 weeks gestation (Group D) on postnatal days 3 to 7 (top row; Group A n=33; Group B n=44; Group C n=29) days 14 to 28 (centre row; Group A n=23; Group B n=19; Group C n=7) and (bottom row) day 3 (Group A n=34; Group B n=44; Group C n=30; Group D n=20) versus day 28 (Group A n=32; Group B n=24; Group C n=9; Group D n=11). Data were analysed using a two-way ANOVA (with repeated measures for line graphs) with the factors gestational age (pGA), postnatal age (pPA) and their interaction (pGAxPA). From Bonferroni post-hoc analysis: *p<0.01 compared to Groups A, B and C (C and I), p<0.05 a versus b and c, b versus c (F and I).

Figure 2: Urine total protein (UTP; A), albumin (B) and β2-microglobulin (β2-M; C) levels corrected for urine creatinine, on postnatal days 7, 14, 21, and 28 in preterm neonates (Groups A n=33, B n=40, and C n=25). Data were analysed using a two-way ANOVA with the factors gestational age (pGA), postnatal age (pPA), and their interaction (pGAxPA).

Figure 3: Urine NGAL levels (A) in neonates born at ≤ 28 weeks gestation (Group A; n=25), 29-31 weeks gestation (Group B; n=20), 32-36 weeks gestation (Group C; n=11) and ≥ 37 weeks gestation (Group D; n=22) at postnatal days 3 and 28. Data were analysed using a two-way ANOVA with the factors gestational age (pGA), postnatal age (pPA) and their interaction (pGAxPA). Linear regression analysis of urinary NGAL versus UTP/Creatinine ratio in preterm neonates, n=68 (B). Urinary NGAL (ng/ml) levels were also assessed in preterm neonates with pathological proteinuria (UTP ≥ 500 mg/L; grey; n=12), with values compared to age and sex-matched controls with normal UTP at the corresponding time point (black; n=12), grouped by gestational age (C). Groups were compared using a two-way ANOVA, with the factors gestational age (pGA), proteinuria (pP) and their interaction (pGAxP). To determine whether NGAL is predictive of proteinuria (D), urinary NGAL levels were assessed in the preterm neonates with
pathological proteinuria (grey; n=12) compared to age- and sex-matched controls (black; n=12) at time points prior to, and at the time of, pathological proteinuria onset. The factors assessed were the time point of assessment (pT), proteinuria (pP) and their interaction (pT \times pP).

Figure 4: The percentage of neonates with impaired renal function (grey) and those with adequate renal function (black) grouped by gestational age (Group A n=33; Group B n=44; Group C n=30, Group D n=22). Impaired renal function (values that differed by more than 2 SD from the mean at any postnatal age, day 3 to day 28) was indicated by low urine output (A), low creatinine clearance (B), high serum creatinine (C), high fractional excretion of sodium (D), high urine total protein (E) or high urinary NGAL (F).
**Table 1:** Pregnancy and birth characteristics of the neonates by gestational age group

<table>
<thead>
<tr>
<th></th>
<th>Group A ≤ 28 weeks (n=33)</th>
<th>Group B 29–31 weeks (n=44)</th>
<th>Group C 32–36 weeks (n=30)</th>
<th>Group D ≥ 37 weeks (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gestational Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(weeks) mean ± SEM</td>
<td>26.6 ± 0.2* (24-28)</td>
<td>30.4 ± 0.1* (29-31)</td>
<td>33.7 ± 0.2* (32-36)</td>
<td>39.6 ± 0.2* (37-42)</td>
</tr>
<tr>
<td><strong>Birth Weight (g)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean ± SEM</td>
<td>811.1 ± 28.3* (529-1229)</td>
<td>1438.0 ± 39.6* (892-2157)</td>
<td>1771.0 ± 75.4* (1018-2542)</td>
<td>3356.0 ± 76.2* (2820-4160)</td>
</tr>
<tr>
<td><strong>Body Length (cm)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean ± SEM</td>
<td>33.6 ± 0.4* (29-39)</td>
<td>40.9 ± 0.4* (36-46)</td>
<td>43.4 ± 0.7* (36-50)</td>
<td>49.9 ± 0.4* (46-55)</td>
</tr>
<tr>
<td><strong>Head Circumference</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(cm) mean ± SEM</td>
<td>23.9 ± 0.2* (21-27)</td>
<td>28.3 ± 0.2* (25-32)</td>
<td>29.9 ± 0.4* (26-33)</td>
<td>34.0 ± 0.3* (32-36)</td>
</tr>
<tr>
<td><strong>Male (%)</strong></td>
<td>36.4</td>
<td>52.3</td>
<td>46.7</td>
<td>50.0</td>
</tr>
<tr>
<td><strong>SGA (%)</strong></td>
<td>30.3†§</td>
<td>9.1†¶</td>
<td>46.7†§</td>
<td>4.5†¶</td>
</tr>
<tr>
<td><strong>Twin/Triplet (%)</strong></td>
<td>27.3†</td>
<td>22.7†</td>
<td>40.0†</td>
<td>0.0*</td>
</tr>
<tr>
<td><strong>Antenatal Steroids</strong></td>
<td>93.8†</td>
<td>94.7†</td>
<td>80.8†</td>
<td>0.0*</td>
</tr>
<tr>
<td><strong>Caesarean</strong></td>
<td>60.6†</td>
<td>67.4†</td>
<td>64.3†</td>
<td>28.6*</td>
</tr>
</tbody>
</table>

Significant differences (P < 0.05) between groups are indicated by symbols: *versus all other groups; †versus Group D; ‡ versus Group C; § versus Group B; ¶ versus Group A.
Table 2: The percentage of neonates with postnatal complications and the percentage exposed to medications during the early postnatal period in each gestational age group

<table>
<thead>
<tr>
<th></th>
<th>Group A ≤ 28 weeks (n=33) (%)</th>
<th>Group B 29 – 31 weeks (n=44) (%)</th>
<th>Group C 32 – 36 weeks (n=30) (%)</th>
<th>Group D ≥ 37 weeks (n=22) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Postnatal complications</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apgar ≤ 7 at 5min</td>
<td>45.5*</td>
<td>25.6*</td>
<td>3.3§¶</td>
<td>0.0§¶</td>
</tr>
<tr>
<td>Mechanical Ventilation</td>
<td>100†‡</td>
<td>84.1†‡</td>
<td>56.7*</td>
<td>0.0*</td>
</tr>
<tr>
<td>Respiratory Distress Syndrome</td>
<td>100†‡</td>
<td>87.2†</td>
<td>50.0†</td>
<td>0.0*</td>
</tr>
<tr>
<td>Culture Positive Sepsis</td>
<td>50.0*</td>
<td>12.2¶</td>
<td>3.3¶</td>
<td>0.0¶</td>
</tr>
<tr>
<td>Intraventricular Haemorrhage</td>
<td>31.3†‡</td>
<td>14.6</td>
<td>0.0¶</td>
<td>0.0¶</td>
</tr>
<tr>
<td>Patent Ductus Arteriosus</td>
<td>68.8*</td>
<td>15.0¶</td>
<td>3.3¶</td>
<td>0.0¶</td>
</tr>
<tr>
<td><strong>Antibiotics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta-Lactam (Benzylpenicillin, Imipenem, Ampicillin)</td>
<td>100†‡</td>
<td>100†‡</td>
<td>80.0*</td>
<td>0.0*</td>
</tr>
<tr>
<td>Aminoglycoside (Gentamicin)</td>
<td>100†‡</td>
<td>100†‡</td>
<td>80.0*</td>
<td>0.0*</td>
</tr>
<tr>
<td>Glycopeptide (Vancomycin)</td>
<td>57.6*</td>
<td>16.7¶</td>
<td>10.0¶</td>
<td>0.0¶</td>
</tr>
<tr>
<td>Macrolide (Erythromycin)</td>
<td>12.5</td>
<td>2.4</td>
<td>3.3</td>
<td>0.0</td>
</tr>
<tr>
<td>Cephalosporin (Cefotaxime, Cefozolin)</td>
<td>12.5</td>
<td>2.4</td>
<td>3.3</td>
<td>0.0</td>
</tr>
<tr>
<td>Nitroimidazole (Metronidazole)</td>
<td>3.1</td>
<td>0.0</td>
<td>3.3</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>Other medications</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antifungal (Nilstat, Nystatin, Fluconazole)</td>
<td>56.3†‡</td>
<td>46.2†‡</td>
<td>14.3§¶</td>
<td>0.0§¶</td>
</tr>
<tr>
<td>Methylxanthine (Aminophylline, Theophylline)</td>
<td>37.5†‡</td>
<td>29.3†</td>
<td>17.9</td>
<td>0.0§¶</td>
</tr>
<tr>
<td>Inotrope (Dopamine, Dobutamine)</td>
<td>28.1*</td>
<td>0.0¶</td>
<td>3.3¶</td>
<td>0.0¶</td>
</tr>
<tr>
<td>Steroid (Hydrocortisone)</td>
<td>3.1*</td>
<td>0.0¶</td>
<td>0.0¶</td>
<td>0.0¶</td>
</tr>
<tr>
<td>Diuretic (Furosemide)</td>
<td>34.4*</td>
<td>7.7¶</td>
<td>3.3¶</td>
<td>0.0¶</td>
</tr>
<tr>
<td>NSAID (Indomethacin)</td>
<td>34.4*</td>
<td>2.3¶</td>
<td>0.0¶</td>
<td>0.0¶</td>
</tr>
</tbody>
</table>

Significant differences (P < 0.05) between groups are indicated by symbols: * versus all other groups; † versus Group D; ‡ versus Group C; § versus Group B; ¶ versus Group A.
Table 3: Measures of renal dysfunction in 12 preterm neonates that exhibited multiple (≥2) measures of renal dysfunction, by gestational age grouping and sex.

<table>
<thead>
<tr>
<th>Gestational Age Group</th>
<th>Sex</th>
<th>Low Urine Output</th>
<th>Low Cr</th>
<th>High Serum Cr</th>
<th>High FE\textsubscript{Na}</th>
<th>High UTP</th>
<th>High NGAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (≤ 28 weeks)</td>
<td>F</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>A (≤ 28 weeks)</td>
<td>F</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>A (≤ 28 weeks)</td>
<td>F</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>A (≤ 28 weeks)</td>
<td>M</td>
<td></td>
<td>✔</td>
<td></td>
<td>✔</td>
<td></td>
<td>✔</td>
</tr>
<tr>
<td>A (≤ 28 weeks)</td>
<td>M</td>
<td></td>
<td>✔</td>
<td></td>
<td>✔</td>
<td></td>
<td>✔</td>
</tr>
<tr>
<td>B (29 – 31 weeks)</td>
<td>M</td>
<td></td>
<td>✔</td>
<td></td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>B (29 – 31 weeks)</td>
<td>F</td>
<td></td>
<td>✔</td>
<td></td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>B (29 – 31 weeks)</td>
<td>M</td>
<td></td>
<td>✔</td>
<td></td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>B (29 – 31 weeks)</td>
<td>M</td>
<td></td>
<td>✔</td>
<td></td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>C (32 – 36 weeks)</td>
<td>M</td>
<td></td>
<td>✔</td>
<td></td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>C (32 – 36 weeks)</td>
<td>F</td>
<td></td>
<td>✔</td>
<td></td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>C (32 – 36 weeks)</td>
<td>M</td>
<td></td>
<td>✔</td>
<td></td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
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</tbody>
</table>