Biomarkers of Kidney Injury in Very-low-birth-weight Preterm Infants: Influence of Maternal and Neonatal Factors

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Abstract. *Background/Aim: Acute kidney injury is an important cause of mortality in very-low-birth-weight (VLBW) preterm infants. As in the general population, the detection of renal damage cannot rely on the measurement of serum creatinine, since it has been demonstrated to be a weak predictor and a delayed indicator of kidney function deterioration. However, several candidate biomarkers have failed to prove sufficient specificity and sensitivity for a routine clinical use because of the poor awareness of their biological role. This study was aimed to investigate the impact of different maternal and neonatal conditions on several renal biomarkers in VLBW preterm infants during the first week of life. Patients and Methods: Preterm infants<32 weeks' gestation and <1500g were enrolled. We measured urinary biomarkers kidney injury molecule 1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), cystatin C, epidermal growth factor (EGF) and osteopontin (OPN) on the 1st, 3rd, and 7th day after birth. Results: Thirty-tree infants were included. The multivariate analysis showed a significant association between gestational age, the presence of patent ductus arteriosus, antenatal maternal hypertension and the levels of urinary biomarkers. Conclusion:*

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There is a possible relation between early biomarkers of renal injury and antenatal, perinatal and post-natal characteristics in VLBW preterm infants during the first week of life.

Preterm birth is burdened by high rates of perinatal morbidity and mortality worldwide (1). With an estimated incidence of 12-18%, acute kidney injury (AKI) is among the main causes of mortality in the preterm population, and represents an independent predictor of mortality even after adjustment for confounding variables, namely comorbidities, interventions and demographic characteristics (2, 3). Preterm birth is also associated with an increased risk of cardiovascular and chronic kidney disease in adulthood; this poses important challenges for the clinicians to detect as soon as possible developmental and functional abnormalities that could affect lifetime (4, 5).

In humans and experimental animals, however, measurable changes in serum creatinine and glomerular filtration rates can be evident only after a remarkable reduction in kidney function (less than 50-80% of normal values), being thus insensitive to detect subclinical renal damage. Moreover, serum creatinine levels can be influenced by several factors, such as gender, age, hydration state, muscle mass, ongoing medications and endogenous metabolites (*e.g.*, bilirubin) (3, 6).

In the neonatal population, AKI definitions based on serum creatinine are susceptible to specific additional limitations: in the first days of life, serum creatinine may reflect the maternal kidney function (7, 8) and it is not able to distinguish pre-renal injuries, which are often reversible and transient, from intrinsic renal injuries (9). Moreover, serum creatinine does not provide specific information on the type of kidney insult (*e.g.*, toxic, infectious, ischemic *etc.*). Hence, several biomarkers other than serum creatinine have been investigated to allow a timely diagnosis of AKI, to localize the site of injury and to predict nephrotoxicity (10). Kidney injury molecule 1 (KIM-1),

neutrophil-gelatinase-associated lipocain (NGAL), interleukin 18 (IL-18), cystatin C, clusterin, fatty acid binding protein-liver type (L-FABP) and osteopontin (OPN) are among the most promising biomarkers proposed for early AKI diagnosis and, in the neonatal population, have the additional benefit of being detected in urine samples, which can be collected non-invasively. In preterm infants, urinary NGAL and epidermal growth factor (EGF) have been shown to predict AKI onset several hours before the rise in serum creatinine (11). However, many candidate biomarkers, taken as single standalone molecules, have failed to prove sufficient specificity and sensitivity for a routine clinical use: this is most likely due to the poor understanding of their biological role (12). Moreover, as for serum creatinine, several conditions may influence its concentration. In adult cohorts, increased urinary and plasmatic NGAL levels have been correlated with the severity of the illness in patients suffering from acute renal failure, but also from other clinical conditions (sepsis, infectious disease, multiple organ failure), and a significant association with mortality rates has been reported (13, 14). Elevated NGAL levels were also found in children with urinary infections (15). Moreover, previous evidence indicated that conditions implying cellular necrosis or apoptosis, such as ischemia or infections, can up-regulate the synthesis of NGAL in different organs (liver, lung and kidney) (16). To date, only few biomarkers have been evaluated in the preterm neonatal population to assess renal function and possibly identify infants at higher risk of AKI. In two nested case-control studies, Askenazi *et al.* explored the individual and combined predictive value of six different urinary biomarkers on AKI and mortality in 123 VLBW infants, observing significantly higher levels of NGAL and OPN in neonates with AKI, and significantly higher OPN and KIM-1 concentrations in non-survivors compared to survivors (17).

In the preterm population, gestational age (GA), birth weight, postnatal age and gender have been reported to significantly affect urine renal biomarkers (18, 19). Hence, in order to contribute to the validation of renal biomarkers for AKI diagnosis in preterm infants, it is crucial to understand the possible role of different antenatal and perinatal factors on their concentration.

This study aimed to investigate the impact of different maternal and neonatal conditions on the changes in kidney injury molecule 1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), cystatin C, epidermal growth factor (EGF) and osteopontin (OPN) as candidate urinary biomarkers of kidney function in VLBW preterm infants during the first week of life.

Patients and Methods

Patients. Infants born at S. Orsola-Malpighi University Hospital, Bologna (Italy) hospital from September 2016 to June 2017 and admitted to the local Neonatal Intensive Care Unit were included in this prospective, observational study if fulfilling the following criteria: GA ≤32 weeks and birth weight <1,500 g. Congenital anomalies of kidney and urinary tract (CAKUT) were exclusion criteria. Infants who died within the first 48 h of life or who developed AKI, defined as an increase in serum creatinine (sCr) ≥0.3 mg/dl by 48 h or ≥1.5 times baseline or urine output below 0.5 ml/kg/h for at least 6 h (20) during the study period were also excluded.

For each infant, the following data were recorded: history of maternal hypertension (MH) prior to delivery as defined by American College of Obstetricians and Gynecologists (21), antenatal evidence of abnormal umbilical flow at Doppler evaluation, maternal chorioamnionitis, GA, birth weight, Apgar score at 1' and 5' (22), clinical risk index for babies II (CRIB II) score (23), gender, twin birth, echocardiographic evidence of an hemodynamically significant patent ductus arteriosus (PDA) over the first week of life, development of medical and/or surgical necrotizing enterocolitis (24) and/or sepsis (defined by clinical signs and symptoms and positive blood culture or C-reactive protein ≥2.5 mg/dl) and need for antibiotics for ≥5 days) (25) during the study period.

The study was approved by the local Ethical Committee (protocol n. 154/2015/U/Oss, BIOPREA-2016) and written informed consent was obtained from the parents or legal carers of the infants.

Assays. Blood and urinary samples were collected on day 1 (T0), day 3 (T1) and day 7 (T2) from the enrolled newborns. Serum creatinine levels (sCr) were determined at the Central Laboratory of our hospital by routine biochemistry methods. Urine specimens were obtained by aspiration from the diaper, and then assayed for urinary biomarkers at the Laboratory of Nephrology, Dialysis and Renal Transplant Unit of the same hospital. Urinary analytes were measured by multiplex methods for simultaneous quantification of different molecules through Luminex MAGPIX technology (Luminex, Austin, TX, USA), and data were analyzed using the Analyst 5.1 PLUS software (Millipore). Three sessions were carried out to determine: (a) NGAL, cystatin C, EGF and OPN; (b) UMOD; (c) calbindin, clusterin, GST-π, IL-18, KIM-1, and MCP-1. The abovementioned urinary biomarkers were assayed using the commercially available kits (MILLIPLEX MAP Human Kidney Injury Magnetic Bead Panel 1 #HKI1MAG-99K and Human Kidney Injury Magnetic Bead Panel 2 #HKI2MAG-99K, Millipore Corp., Billerica, MA; Bio-Plex Pro™ RBM Human Kidney Toxicity Assays, Bio-Rad Laboratories, Segrate, Italy). The concentrations were calculated using a standard curve through serial dilutions. For the aim of the study, only KIM-1, NGAL, cystatin C, EGF and OPN were analyzed. The sensitivity limits (minimum detectable concentrations) were 0.014 ng/ml for KIM-1, 0.01 ng/ml for NGAL, 0.16 ng/ml for cystatin C, 0.02 ng/ml for EGF and 0.40 ng/ml for OPN. The coefficients of variation (CV%) were: <10% intra-assay and <15% inter-assay for KIM-1, NGAL and OPN; <10% intra-assay and <20% inter-assay for cystatin C; <10% intra-assay and <10% inter-assay for EGF.

Statistical analysis. The distribution of renal biomarkers was determined by means of Shapiro-Wilk test. Since these parameters followed a normal distribution, differences over time (T0, T1 and T2) in the renal biomarkers were evaluated by one-way ANOVA. Student's *t*-test was used to compare renal biomarkers at T0, T1 and T2 in relation to different maternal and neonatal variables. A multiple linear regression model, including AKI biomarkers at different time points as dependent variables and the factors that resulted statistically significant at the univariate analysis as independent variables, was built to evaluate the independent effect of each variable on the observed results. The significance level was set at $p<0.05$. All the analyses were conducted using IBM SPSS, version 25.0 (SPSS Inc., Chicago, IL, USA).

Table I. *Clinical characteristics of the study population*.

Clinical characteristics	N=32
Gestational age (weeks), median (interquartile range, IQR)	29 (26.5-30)
Birth weight (g), median (IQR)	1092 (826-1,363)
Apgar score	
1', median (IQR)	8 (7-8)
5', median (IQR)	9 (9-9)
CRIB II score	
Level 1, n (%)	9 (28.1)
Level 2, n (%)	16 (50)
Level 3, n (%)	7 (21.9)
Level 4, n (%)	0 (0)
Maternal age (years), median (IQR)	36 (31-39)
Maternal hypertension, n (%)	7 (21.9)
Antenatal umbilical Doppler	
Normal, n (%)	24 (75)
Absent or reversed end-diastolic flow, n (%)	8 (25)
Gender	
Males, n (%)	24 (75)
Females, n (%)	8 (25)
Type of gestation	
Twin, n (%)	13 (59.4)
Single, n (%)	19 (40.6)
Mode of delivery	
Vaginal delivery, n (%)	5 (16)
Caesarean section, n (%)	27 (84)
Status of ductus arteriosus	
Patent, hemodynamically significant, n (%)	11 (34.4)
Restrictive or closed, n (%)	22 (65.6)

CRIB: Clinical risk index for babies.

Results

Thirty-three VLBW infants were enrolled in the study. The clinical characteristics of the study population are shown in Table I. Out of 8 infants with antenatal impairment of umbilical Doppler, 7 had a maternal history of hypertension prior to delivery.

Trends over time of sCr, KIM-1, NGAL, cystatin C, EGF and OPN are illustrated in Figure 1. No changes in sCr were observed from T0 to T2. Although not significant, a trend towards a decrease from T0 to T2 was found for cystatin C and KIM-1, whereas EGF and NGAL showed a slightly increased excretion at T1.

As shown in Table II, GA, PDA and antenatal MH were associated with significantly different levels of all the studied biomarkers at different time-points over the first week of life. Infants with GA below 30 weeks showed significantly increased levels of NGAL and cystatin C from T0 to T2 and higher sCr at T1 and T2, while no differences were observed for KIM-1 and EGF. Moreover, newborns with PDA displayed significantly higher values of sCr from T0 to T2, KIM-1 at T0 and cystatin C, OPN, NGAL at T2. There were no significant

differences between genders at any of the experimental times, apart from a tendency towards higher NGAL values in females at T0, T1 and T2. A history of antenatal MH was associated with a significant increase in serum creatinine at T0 and T1 and higher NGAL and cystatin C levels at T0.

Given the intrinsic association between MH, GA and PDA, a multiple linear regression model was developed to assess the independent effect of each variable at specific time points. According to the model, a significant effect of GA was also confirmed for sCr at T1 (B=0.145, *p*=0.020) and T2 (B=0.321, *p*=0.012), NGAL at T1 (B=199.92, *p*=0.035), and cystatin C at T0 (B=144.62, *p*=0.004), whereas the presence of PDA was independently associated with a higher sCr at T0 (B=0.189, *p*=0.004). Moreover, a significant impact of antenatal MH on sCr at T1 (B=0.156, *p*=0.036) and on NGAL at T0 (B=182.45, *p*=0.014) and T1 (B=198.04, *p*=0.043) was confirmed.

No history of maternal chorioamnionitis was documented. Moreover, none of the enrolled infants had a CRIB II score >15, nor developed sepsis or necrotizing enterocolitis during the first week of life.

Discussion

According to our results, different neonatal and maternal factors can influence renal biomarkers in VLBW preterm neonates without AKI over the first week of life. In particular, GA, the presence of PDA and antenatal MH were significantly associated with the levels of sCr, NGAL, and cystatin C at specific days after birth.

NGAL is certainly the most studied biomarker as an early predictor of AKI in several clinical settings, although data from preterm infants are still scanty. We have previously reported that renal function in VLBW infants is associated with urinary NGAL levels in the first 3 days of life (12). Similarly, Argyri *et al.* reviewed the possible diagnostic role of some promising biomarkers in the early diagnosis and prognosis of AKI in newborns (26). Indeed, how different pathophysiological factors or maternal and neonatal characteristics may affect the changes in these biomarkers has not been fully clarified yet. Here, we observed a significant inverse correlation between GA and NGAL concentration during the first week of life. Our data are thus consistent with the findings by Lavery *et al.* who found a negative association between NGAL and birth weight, which can be regarded as a proxy for GA (27). This GA-dependency of NGAL values is feasibly due to the immature tubular function ensuing from preterm birth. The evidence of a progressive decline in urinary NGAL in preterm-born neonates over the first 3 months of life could reflect the progressive post-natal maturation of nephrogenesis, which is complete around 36 weeks post-conceptional age (28).

In a prospective cohort study, Saeidi *et al.* evaluated 14 urine biomarkers in 81 preterm infants without AKI,
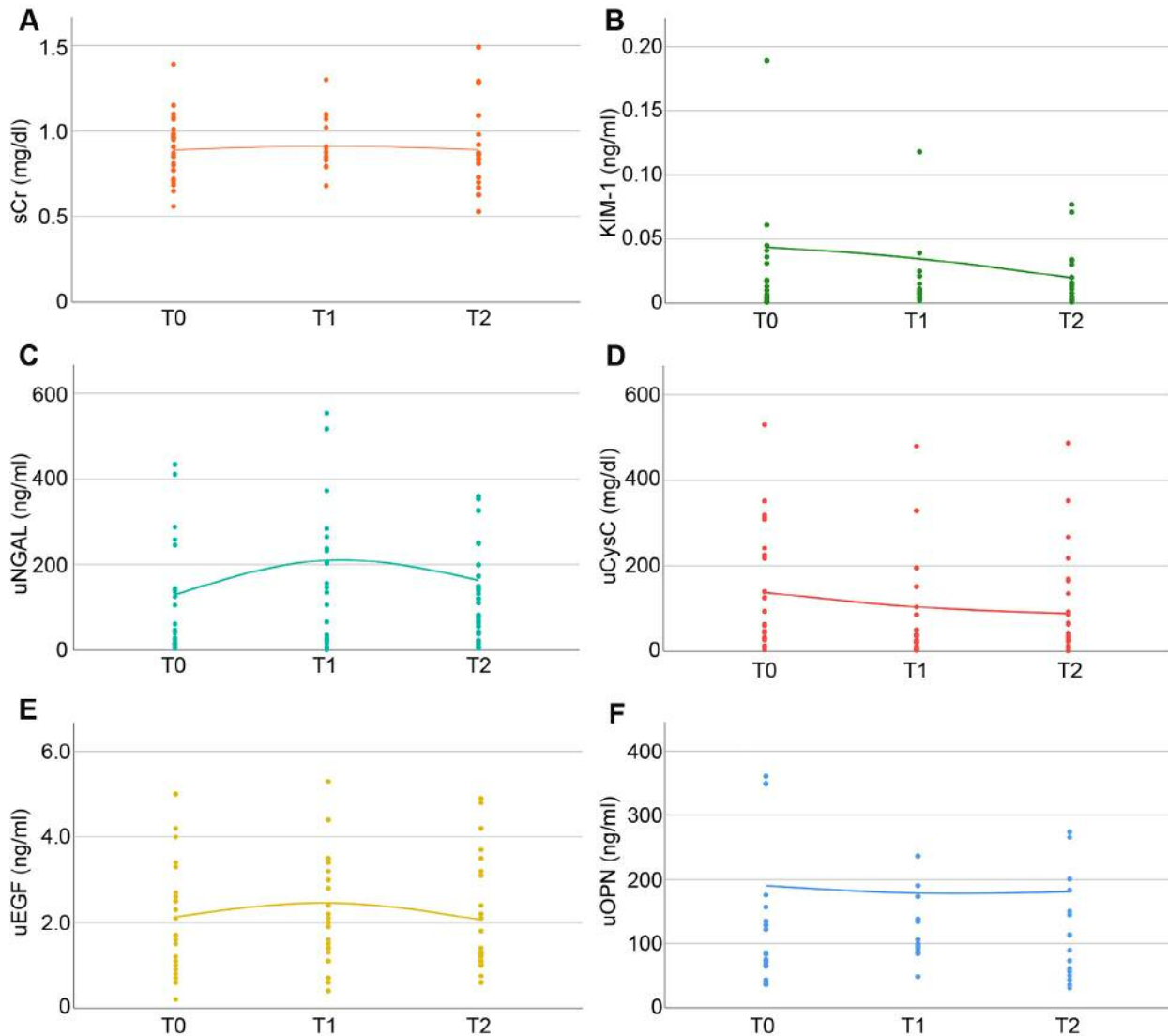
Figure 1. *Levels of serum creatinine (A) and urinary KIM-1 (B), NGAL (C), cystatin C (D), EGF (E) and OPN (F) at T0 (day 1), T1 (day 3) and T2 (day 7). EGF: Epidermal growth factor; KIM-1: kidney injury molecule 1; NGAL: neutrophil gelatinase-associated lipocalin; OPN: osteopontin; sCr: serum creatinine.*

observing a significant association between the concentration of biomarkers (in particular, NGAL and cystatin C) and clinical variables as postnatal age, GA and gender (17). Consistently, DeFreitas *et al*. found a significant association between cystatin C and GA (29). The progressive decrease in urinary NGAL excretion observed over the first 4 days of life was not confirmed by our results.

Concerning PDA, up to now, only a few studies have evaluated its impact on renal biomarkers. Tosse *et al*. found higher uNGAL levels in 37 neonates with a birth weight below 1,500 g and with a PDA that needed either medical or surgical intervention, compared to matched controls with no PDA or a PDA requiring no intervention (30). In our cohort, the presence of PDA seemed to increase the levels of some biomarkers.

However, it should be recognized that 10 out of 11 neonates with evidence of PDA during the first week of life were treated with ibuprofen; hence, it was not possible to discriminate the effect of PDA from that of the related drug therapy on renal biomarkers.

More recently, Sellmer *et al*. evaluated the impact of PDA on a cohort of 146 neonates with GA below 32 weeks; after adjusting for GA and gender, no difference in relation to the PDA status was observed, thus suggesting that NGAL may not be influenced by PDA (31). In line with this study, in our infants we found a significant correlation between the levels of renal biomarkers and the presence of PDA at the univariate analysis, but this finding was not confirmed by the multivariate model, where the effects of other covariates (*i.e*., GA, MH) prevailed over PDA in determining NGAL concentration.

Table II. *Between-group comparison of renal biomarkers at T0 (day 1), T1 (day 3) and T2 (day 7) in relation to the infants' gestational age, ductal status, history of maternal hypertension and gender.*

T0	GA<30 wks	GA≥30 wks	PDA	No PDA	HM	NM	Males	Females
sCr (mg/dl)	0.95	0.82	0.98**	0.78**	0.98*	0.83*	0.90	0.91
	(0.79-0.99)	(0.71-0.91)	(0.96-1.04)	(0.71-0.87)	(0.93-1.09)	(0.72-0.96)	(0.74-0.97)	(0.79-1.01)
KIM-1 (ng/ml)	0.011	0.018	0.040*	0.01*	0.036	0.015	0.015	0.031
	(0.004-0.063)	(0.01-0.036)	(0.008-0.103)	(0.001-0.018)	(0.007-0.045)	(0.003-0.038)	(0.004-0.044)	(0.005-0.036)
NGAL (ng/ml)	136.1*	15.4*	124.6	35.5	249.3**	69.1**	33.7	133.7
	(39.3-258.3)	(7.7-39.2)	(39.3-245.7)	(12.9-117.2)	(115.1-515.9)	(37.6-142.7)	(15.7-137)	(57.1-318.4)
CysC (mg/dl)	218.1**	29.4**	224.6	44.1	241*	45.7*	60.4	143.7
	(62-310.3)	(15.9-32.8)	(62-308.6)	(30-127.9)	(143.3-309.4)	(30.6-128.5)	(30.7-178.8)	(37.1-312.1)
EGF (ng/ml)	2.1	2.1	2.1	1.65	2.3	1.65	2.1	1.85
	(1.1-2.5)	(1-3.1)	(1.7-3.4)	(1.02-2.57)	(2.1-2.4)	(0.97-2.85)	(1.2.55)	(1.25-3.7)
OPN (ng/ml)	128.7*	65.3*	85.7	77.3	262.3	71.6	85.7	75
	(84.4-392.7)	(42.4-74.1)	(75-121.8)	(56.4-161.7)	(162.1-370.8)	(43-128.7)	(66.3-166.3)	(36.3-128.7)

T1	GA<30 wks	GA≥30 wks	PDA	No PDA	HM	NM	Males	Females
sCr (mg/dl)	0.90*	0.81*	1.04*	0.84*	1.07*	0.85*	0.85	0.88
	(0.86-1.07)	(0.79-0.84)	(0.89-1.09)	(0.79-0.88)	(1.02-1.1)	(0.79-0.88)	(0.79-0.91)	(0.85-1.02)
KIM-1 (ng/ml)	0.009	0.011	0.01	0.009	0.01	0.011	0.013	0.006
	(0.007-0.016)	(0.004-0.025)	(0.008-0.021)	(0.006-0.026)	(0.003-0.013)	(0.007-0.027)	(0.008-0.029)	(0.003-0.008)
NGAL (ng/ml)	218.7*	23.2*	85.7	105.8	372.4*	120.3*	105.8	186.1
	(143.2-413.1)	(6-65.6)	(20-459.3)	(18.9-251)	(118.4-590.8)	(23.9-225.2)	(22.9-218.8)	(61.1-396.6)
CysC (mg/dl)	85.6	23.1	77.9	30.5	85.6	35.1	20.7	55.8
	(38.4-172.9)	(20.4-46.3)	(33.9-115.2)	(20.8-59.7)	(50-150.9)	(20.6-69.6)	(9.6-50.2)	(10.7-150.9)
EGF (ng/ml)	2.3	2	2.2	2.55	1.5	2.6	2.2	2.7
	(1.4-3.5)	(1.6-3.4)	(1.4-3)	(1.45-3.72)	(1.35-2.85)	(1.6-73.47)	(1.4-3.35)	(1.5-3.7)
OPN (ng/ml)	106.2	91.8	164.1*	96.2*	97.1	99.5	106.2	64.7
	(92.7-190.1)	(57.3-125.4)	(100.6-224.9)	(84.1-106.2)	(87.3-122.9)	(88.4-164.1)	(88.1-173.2)	(25-231.8)

T2	GA<30 wks	GA≥30 wks	PDA	No PDA	HM	NM	Males	Females
sCr (mg/dl)	0.89*	0.67*	0.92*	0.77*	1.05	0.85	0.85	0.95
	(0.85-1.13)	(0.63-0.7)	(0.86-1.21)	(0.66-0.88)	(0.75-1.28)	(0.7-0.9)	(0.67-0.95)	(0.77-1.19)
CysC (mg/dl)	75.8*	26.9*	91.8*	30*	62.9	34.3	33.8	39.3
	(35.6-167.6)	(22.7-30)	(38.3-193.2)	(23-3-40)	(18.9-179.5)	(27.2-80.9)	(25.3-134.6)	(29.9-64.3)
KIM-1 (ng/ml)	0.006	0.016	0.008	0.005	0.011	0.008	0.013	0.003
	(0.004-0.013)	(0.007-0.029)	(0.013-0.016)	(0.005-0.023)	(0.005-0.016)	(0.005-0.017)	(0.005-0.024)	(0.002-0.005)
NGAL (ng/ml)	142.7*	42.6*	287.7**	59.3**	245.7**	41.1**	79.6	140.1
	(81.6-326.1)	(12.8-70.4)	(125.9-357.3)	(26.4-128.5)	(9.7-422.3)	(15.6-127.5)	(33.9-150.1)	(66.2-276.2)
EGF (ng/ml)	1.6	2.1	1.8	1.75	1.4	1.95	1.3	3.1
	(1.2-3.6)	(1.2-2.6)	(1.3-3.6)	(1.12-2.37)	(1.2-3.45)	(1.21-2.92)	(1.2-2.2)	(1.7-3.4)
OPN (ng/ml)	144.8	93.3	265.6**	50.3**	119.8	67	75.1	93.3
	(50.3-265.6)	(63.9-113-3)	(192-454.6)	(15.4-93.3)	(81.3-158.4)	(31.9-186.7)	(38.1-196.3)	(36.8-129.1)

*p<0.05; **p<0.001. CysC: Cystatin C; EGF: epidermal growth factor; GA: gestational age; HM: hypertensive mother; KIM-1: kidney injury molecule 1; NGAL: neutrophil gelatinase-associated lipocalin; NM: normotensive mother; OPN: osteopontin; PDA: patent ductus arteriosus; sCreat: serum creatinine; T0: post-natal day 1; T1: post-natal day 3; T2: post-natal day 7.

Besides, we observed significantly higher levels of urinary NGAL during the first 3 days of life in case of antenatal history of MH; this finding was confirmed even after adjustment for other covariates.

Hypertensive disorders are a major complication of pregnancy and represent one of the leading causes of maternal and fetal morbidity and mortality (32). Hypertension is a multifactorial disease involving endothelial cells and leads to a variety of clinical symptoms including proteinuria and systemic inflammation (33). In a prospective case-control study, Patel *et al*. compared serum NGAL between three groups (mild preeclampsia, severe preeclampsia, and eclampsia) and controls. According to their results, NGAL levels significantly differed between the study groups, with highest values in eclamptic women, followed by those with severe and mild preeclampsia (34). In this study, we found higher serum levels of NGAL in women with severe pre-eclampsia compared to controls, although they did not reach

statistical significance, and a significant correlation between serum NGAL and the levels of proteinuria in pre-eclamptic women (35). These findings support a possible implication of NGAL in hypertension-related inflammatory processes. As such, the higher urinary NGAL levels observed in infants with MH seem to indicate that hypertensive disorders of pregnancy might trigger a pro-inflammatory status not only in the mother, but also in the offspring. In support of this hypothesis, elevated pro-inflammatory molecules in the cord blood of neonates born by hypertensive mothers compared to normotensive ones have been previously reported (36).

Our data indicate that clinical factors other than GA may influence the early levels of renal biomarkers, including NGAL. As for sex, higher NGAL levels in females compared to males have been reported (17); in this study, females had slightly higher NGAL values at each of the three study evaluations. Regarding clinical antenatal and neonatal conditions potentially influencing renal biomarkers, a previous study on VLBW infants reported increased NGAL levels in the presence of culture-positive sepsis (37), suggesting a possible role for this biomarker in the prediction of late onset sepsis, given its rapid urinary excretion. However, we did not evaluate this point, since none of our infants developed sepsis during the period of evaluation.

The small sample size represents the main limitation of this study. Furthermore, given the low number of infants in the highest CRIB II classes, it was not possible to investigate the possible association between urinary biomarkers and CRIB score, an important predictor of mortality in preterm infants. Eventually, the disproportionate ratio in favour of males over females may have biased the power of the gender comparison that, differently from other studies, has led to non-significant results.

In conclusion, our data suggest the existence of a complex relationship between early renal biomarkers and antenatal, perinatal and post-natal characteristics in healthy preterm infants during the first week of life. Larger targeted studies are needed to validate these findings and to better clarify the physio-pathological mechanisms influencing renal biomarkers, in order to improve their predictive value on AKI detection in the premature neonatal population.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

I.C., main protocol design and preparation of the manuscript; F.V., S.M., S.G., E.P. protocol design; F.Z., V.A. collection of specimens and data, contribution to manuscript preparation; C.D. laboratory assays, database update; M.C., laboratory assays, data collection, manuscript final revision; L.L., A.G. contribution to protocol design, statistical analysis; G.F., F.D.P., GLM, main supervisors.

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