Evolution of the urinary proteome during human renal development and maturation: variations with gestational and postnatal age

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BACKGROUND: Low birth weight is associated with deficits in nephron number in the infant kidney and increased risk of adulthood hypertension and renal dysfunction. Urinary biomarkers may be potential indicators of renal reserve, but little is known about the influence of gestational and postnatal age on the expression of urinary proteins. The aims of this study were to determine the relationships between selected urinary proteins and renal maturation. We hypothesized that urinary protein patterns would change over time during late nephrogenesis and renal maturation.

METHODS: Urine samples were collected at birth and over 12 mo from preterm (33–35 wk) and term (38–40 wk) infants. Candidate urinary proteins were identified by antibody array and quantified with enzyme-linked immunosorbent assay.

RESULTS: Preterm infants at birth were found to have relatively elevated levels of insulin-like growth factor binding protein-1, -2, and -6, monocyte chemotactic protein-1, CD14, and sialic acid-binding Ig-like lectin 5. These markers gradually decline to levels similar to those of full-term infants by 2–6 mo of life. In contrast, many urinary markers in healthy full-term infants remain stable over the first year of life.

CONCLUSION: Gestational and postnatal age must be considered when evaluating the utility of urinary biomarkers.

Premature infants are challenged to adapt to an *ex utero* environment while organogenesis remains incomplete. In humans, ~60% of nephrons develop during the third trimester (1) with nephrogenesis complete at 32–36 wk gestation (2). Although the kidney continues to mature for an additional 1–2 y, full-term infants have a complete endowment of nephrons, varying widely from 250,000 to 1.8 million per kidney, by 36 wk gestation (2,3) and do not form new nephrons after birth. However, the duration of nephrogenesis and the health of the nephrons in infants born prematurely remain unknown. There appears to be a correlation between gestational age and nephron number in large animal species. Studies in baboons demonstrate a direct correlation between gestational age and nephron number (4). Moreover, prematurely delivered baboons continue

to develop nephrons after birth, but the length of gestation inversely correlates with the number of abnormal nephrons (4) (the lower the gestational age the higher the number of abnormal nephrons). There may be a limited window for nephrogenesis to continue postnatally in humans. A single autopsy study has shown that nephrogenesis appears to continue for 40 postnatal days in extremely preterm human infants and then cease (5). A smaller complement of nephrons may not be reflected by renal dysfunction in the preterm infant. However, adults born at a low birth weight, with a reduced number of nephrons, are at increased risk of hypertension, impaired kidney function, and cardiovascular disease (6).

As the overall mortality of the premature population decreases, the risk of long-term morbidity in this population emerges (7). Barker and Martyn (8) established a framework for the intrauterine origin of disease. They associated fetal malnutrition with adult diseases, demonstrating that low birth weight in infancy correlates with earlier morbidity and mortality as adults (8). Brenner *et al.* (9) extended this hypothesis with observations that hyperfiltration and hypertrophy of reduced numbers of nephrons leads to hypertension and progression of chronic kidney disease. Therefore the kidneys of premature infants may suffer permanent effects of early exposure to the extra-uterine environment manifesting as renal disease in adulthood, in part because of reduced nephron reserve. In recent years, infants with a birth weight <2.5 kg have been highlighted as a particularly vulnerable population for adult disease given their likelihood of reduced nephron number (10). In 2009, a metaanalysis reported that infants with birth weights <2.5 kg have a 70% increased risk of chronic kidney disease as they reach adulthood, and the National Kidney Foundation now recognizes low birth weight as a risk factor for chronic kidney disease (11,12).

In clinical practice, it is difficult to recognize the signs of nephron deficit or dysfunction until the quantity of nephrons is dramatically reduced. The most widely available index of renal function is serum creatinine concentration. Unfortunately, creatinine concentration is a relatively poor marker for monitoring

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renal disease, increasing only when >50% of nephrons are nonfunctional (13). Presently, there are no methods to identify which preterm infants are at highest risk for chronic kidney disease. Given the continued increase in the number of infants born prematurely and the number and costs of adults with chronic kidney, it has become imperative to discover markers to evaluate renal health during renal development and maturation and to identify children at high risk for the development of lifelong chronic kidney disease.

Analysis of urinary proteins provides a unique window into dynamically changing renal developmental processes. Urine is easy to handle, noninvasive to obtain, and provides information about the whole organ. Moreover, 70% of urinary proteins estimated to originate from the kidney and urinary tract (14). Data exist on a few individual markers, but little is known about how they play a role in the developmental and maturational processes in infants. The aims of this study were to determine the relationship between selected urinary proteins and renal maturation. We hypothesized that urinary protein patterns would change over time during late nephrogenesis and early postnatal renal maturation.

RESULTS

Characteristics of Infant Groups

Gestational age, birth weight, antibiotic exposure, mode of delivery, and length of stay were compared in preterm and full-term infant groups (Table 1). Birth weight, gestational age, and length of stay were statistically different between the preterm and term groups. The median birth weight of premature infants was <2.5 kg.

Selection of Candidate Urinary Markers

After analysis of the G2000 array, 20 of the 174 proteins were deemed candidate markers; 17 of 20 with a relative fluorescent intensity (RFI) measurement >1,000 and 3 of 20 in the <1,000 range. The most dramatic differences in urinary cytokine levels between the groups were demonstrated in the preterm group at birth as compared with the term group at birth. Sixty-six of 174 proteins were detected in either group at any time point at a level of >200 RFI. The remaining 110 proteins on the array were undetectable by this technique. The 20 chosen candidate markers are displayed in Table 2 illustrating the urinary protein excretion patterns comparing each group at the two selected time points: birth and 6 mo and over time in both the preterm and full-term groups. From this list of 20 candidate markers, six were chosen to validate (insulin-like growth factor (IGF)-II, IGF binding protein

(IGFBP)-1 and -6, monocyte chemotactic protein-1(MCP-1), CD14, and sialic acid-binding Ig-like lectin 5 (Siglec-5)). Five additional markers, found on the large array but not significant between the groups (IGF-I, IGFBP-2, 3, 4, 5), were added to the quantification studies to more thoroughly evaluate the components of the IGF axis. Markers that would require dilution were not chosen to quantify as dilution of the sample would decrease the potential to detect a signal from other less abundant proteins.

Of the 11 urinary markers quantified using the customized multiplex enzyme-linked immunosorbent assay, no informative data was obtained on five: IGF-I, IGF-II, IGFBP-3, IGFBP-4, and IGFBP-5. The standard curve for IGF-II proved unreliable. The concentrations of IGF-I, IGFBP-3, IGFBP-4, and IGFBP-5 were below detection for the majority of time points analyzed in both term and preterm infants, confirming the initial array findings. Additional analysis of these largely unmeasurable data using a binary outcome of whether or not the value was above zero, continued to show no statistical pattern discernible within the data.

Preterm Infants Compared With Term Infants

Initial identification of urinary biomarkers. At birth, 15 of 20 candidate cytokines were significantly higher in the preterm group as compared with the term group (Table 2). These markers included angiogenin, epidermal growth factor, interferon- γ , IGFBP-1, interleukin (IL)-1 α , IL-3, MCP-1, tumor necrosis factor- β , CD14, IGF-II, platelet derived growth factor AA, Siglec-5, nerve growth factor receptor, granulocyte colony-stimulating factor, and IGFBP-6. By 6 mo, there was no statistically significant difference in the levels of any of the 20 candidate markers between the preterm and full-term groups (Table 2).

Quantitation studies. As seen in Figure 1, urinary levels of IGFBP-1, IGFBP-6, MCP-1, Siglec-5, and CD14 were confirmed as significantly higher in the preterm infants at birth as compared with the full-term infants. With the exception of IGFBP-2, the pattern of excretion of all of the urinary markers was similar. Each urinary marker was relatively elevated in the preterm infants at birth and decreased over time: by 6 mo of age, the urinary levels of the six selected markers were indistinguishable between the term and preterm groups.

Each marker in the preterm groups normalized to the values of the term infants at a different time point, between 2 and 6 mo of age. Both IGFBP-1 and MCP-1 were higher in the preterm group until the age of 6 mo (Figure 1a,d). As seen in

Table 1. Baseline characteristics of the preterm and full-term infant groups

	Median gestational age (range)	Median birth weight (range)	Number receiving antibiotics	Number born via C-section	Median length of stay (range)
Preterm infants, <i>n</i> = 13	34.00 wk* (33.00–35.71)	2,140 g* (1,640–2,895)	6	4	14 d* (2–25)
Full-term infants, <i>n</i> = 13	39.57 wk (38.29–40.70)	3,539 g (2,549–3,810)	1	6	3 d (2–4)

The median gestational age, birth weight, and length of hospitalization with ranges for the preterm and full-term infant groups that were used in the quantification studies. Birth weight (*t*-test, *P* < 0.001), gestational age (Mann–Whitney rank sum, *P* < 0.001), and length of stay (Mann–Whitney rank sum, *P* = 0.003) were statistically different between the preterm and term groups.

**P* value < 0.05.

Table 2. Twenty candidate markers selected and compared between groups: birth (preterm vs. term), 6 mo (preterm vs. term), preterm group over time (birth vs. 6 mo), and term group over time (birth vs. 6 mo)

Protein	Birth			6 mo			Birth vs. 6 mo	
	Preterm median ^e (range)	Term median ^e (range)	Mann–Whitney <i>P</i> value	Preterm median ^e (range)	Term median ^e (range)	Mann–Whitney <i>P</i> value	Preterm ^b	Term ^c
							Wilcoxon <i>P</i> value	
Angiogenin	65 (29–1,142)	2 (0–82)	0.03	50 (3–544)	231 (14–1,157)	0.325	0.148	0.008
EGF	4,687 (2,026–20,911)	766 (431–3,674)	<0.001	4,021 (3,115–28,733)	3,691 (1,579–15,363)	0.442	0.844	0.023
IFN- γ	110 (17–422)	30 (1–128)	0.021	136 (10–1,819)	107 (30–607)	0.721	1	0.016
IGFBP-1 ^a	1,545 (469–7,467)	255 (1–128)	<0.001	311 (85–537)	272 (173–897)	0.959	0.023d	0.547
IL-1 α	54 (22–133)	12 (3–33)	<0.001	77 (30–342)	48 (21–147)	0.328	0.313	0.016
IL-3	473 (41–1,395)	147 (5–488)	0.021	521 (33–6,410)	499 (69–2,326)	0.878	1	0.016
IL-6	168 (14–695)	71 (1–205)	0.065	251 (8–2,644)	168 (23–700)	0.721	0.742	0.023
Leptin	184 (15–554)	53 (0–272)	0.08	228 (2–2,092)	151 (19–778)	0.721	0.945	0.055
MCP-1 ^a	198 (16–896)	9 (2–225)	0.003	320 (26–456)	130 (45–341)	0.13	0.945	0.008
TNF- β	67 (16–172)	13 (1–52)	0.003	62 (10–644)	52 (22–233)	0.645	0.844	0.016
Acrp30	34 (2–2,344)	8 (2–338)	0.161	59 (5–913)	16 (3–602)	0.279	0.844	0.078
ICAM-3	4 (1–1,433)	1 (0–35)	0.13	22 (1–489)	4 (1–734)	0.645	0.547	0.039
CD14 ^a	437 (99–1,647)	68 (14–298)	0.002	219 (10–447)	142 (79–384)	0.878	0.008d	0.109
IGF-II ^a	265 (50–7,151)	56 (11–208)	0.038	421 (48–2,357)	400 (13–3,811)	0.949	0.844	0.055
MMP-9	21 (0–320)	1 (0–70)	0.105	92 (0–408)	54 (10–523)	0.798	0.461	0.008
PDGF AA	62 (9–538)	2 (0–82)	0.002	38 (4–185)	54 (8–305)	0.645	0.148	0.016
Siglec-5 ^a	2,028 (5–5,306)	18 (1–249)	0.021	11 (7–343)	29 (2–44)	0.505	0.016d	0.547
NGF-R	44 (16–107)	10 (4–34)	0.016	54 (26–143)	50 (14–115)	0.382	0.547	0.016
GCSF	126 (28–174)	24 (4–92)	0.008	96 (17–885)	83 (37–247)	0.798	1	0.016
IGFBP-6 ^a	23 (6–377)	2 (1–8)	0.001	13 (5–54)	9 (3–72)	0.328	0.383	0.016

Acrp30, adipocyte complement-related protein of 30 kDa; EGF, epidermal growth factor; GCSF, granulocyte colony-stimulating factor; ICAM-3, intercellular adhesion molecule-3; IFN- γ , interferon γ ; IGF-II, insulin-like growth factor 2; IGFBP, insulin-like growth factor binding protein; IL-1 α , interleukin-1- α ; IL-3, interleukin-3; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; MMP-9, matrix metalloproteinase-9; NGF-R, nerve growth factor receptor; PDGF AA, platelet derived growth factor AA; Siglec-5, sialic acid-binding Ig-like lectin 5; TNF- β , tumor necrosis factor- β .

^aIGFBP-1, MCP-1, CD14, IGF-II, Siglec-5, and IGFBP-6 were chosen to quantify. ^b“Preterm” compares the preterm group over time (birth vs. 6 mo). ^c“Term” compares the term group over time (birth vs. 6 mo). ^dIGFBP-1, CD14, and Siglec-5 decreased significantly in the preterm group from birth to 6 mo. ^eUnits of measurement are relative fluorescent intensity/urine creatinine (mg/dl).

Figure 1b, the urinary excretion of IGFBP-2 in preterm infants as compared with the term infants at birth did not reach statistical significance. However, the trend is similar to those of the other markers. Although urinary IGFBP-2 excretion is not higher in the preterm infants at birth, it is higher than term infant excretion at 4 wk and 2 mo. By 6 and again at 12 mo, there was no difference in the urinary excretion of IGFBP-2 between preterm and full-term infants.

The pattern of IGFBP-6 (**Figure 1c**), Siglec-5 (**Figure 1e**) and CD14 (**Figure 1f**) is the same: the preterm infants have higher levels than the term infants at birth and at 4 wk, but by 2 and up to 12 mo they have levels indistinguishable from the term infants.

Preterm Group Over Time

Initial identification of urinary biomarkers. Three of 20 candidate cytokines (**Table 2**) decreased significantly in the

preterm group from birth to 6 mo of age (IGFBP-1, CD14, and Siglec-5); no candidate markers increased over time in this group.

Quantitation studies. The preterm group (black dashed line, **Figure 1**) demonstrated a significant decrease in the urinary concentrations of IGFBP-1, IGFBP-2, IGFBP-6, MCP-1, Siglec-5, and CD14 from birth to 12 mo.

Term Group Over Time

Initial identification of urinary biomarkers. The discovery array identified 14 proteins (**Table 2**) that increased significantly in full-term infants over the time from birth to 6 mo. These included: angiogenin, epidermal growth factor, interferon- γ , IL1 α , IL-3, IL-6, MCP-1, tumor necrosis factor- β , intercellular adhesion molecule-3, matrix metalloproteinase-9, platelet-derived growth factor AA, nerve growth factor

Figure 1. Urinary biomarkers in preterm and full-term infants over 12 mo. Urinary concentrations of the markers are elevated in preterm as compared with full-term infants at birth, but by 2 mo of age insulin-like growth factor binding protein (IGFBP)-6, sialic acid-binding Ig-like lectin 5 (Siglec-5), and CD14 had normalized and there is no difference in IGFBP-1, IGFBP-2, or monocyte chemotactic protein (MCP)-1 between the groups by 6 mo of age. Over time there was a significant decrease in all urinary markers in the preterm infants, but no change in the term group. Both birth and 4-wk urine samples in the preterm group were compared with the full-term infant samples. (a) Urinary IGFBP-1, (b) urinary IGFBP-2, (c) urinary IGFBP-6, (d) urinary MCP-1, (e) urinary Siglec-5, and (f) urinary CD14. *Preterm (–1 mo = birth) vs. term (0 mo = birth) $P < 0.01$; **preterm (–1 mo = birth) vs. term (0 mo = birth) $P < 0.05$; †preterm vs. term at y mo $P < 0.01$ (y = 0, 2, 6, 12); ‡preterm vs. term at y mo $P < 0.05$ (y = 0, 2, 6, 12); §IGFBP-6 is higher in the term group as compared with preterm group at 6 mo.

receptor, granulocyte colony-stimulating factor and IGFBP-6; no detectable proteins decreased.

Quantitation studies. Figure 1 illustrates the relative stability of urinary levels in term infants over time. From birth to 6 mo the only marker that increased significantly was IGFBP-6 (P value = 0.03). The levels of IGFBP-1, IGFBP-2, MCP-1, Siglec-5, and CD14 did not change from birth to 6 mo, nor from birth to 12 mo.

DISCUSSION

This study is the first to measure and compare over time the abundance of various urinary proteins in the same preterm and full-term infants. Selected urinary proteins (IGFBP-1, -2, -6, MCP-1, CD14, and Siglec-5) analyzed by an antibody array are higher in healthy preterm infants born only a few weeks before the completion of gestation, as compared with full-term infants at birth. This finding is particularly significant given the heterogeneous characteristic of the preterm group, recruited from both the neonatal intensive care unit (NICU) and newborn nursery. Antibiotics and other medications, as well as clinical variables could theoretically alter the pattern of urinary proteins detected. The results could not be stratified for these variables because of the small sample size. Despite the variability in the preterm group, over time, the levels of

the markers clearly decline in the urine of preterm infants, although urinary excretion of these markers was relatively stable in the full-term infants from birth to 12 mo. Therefore, our results suggest that as renal maturation continues, excretion of urinary proteins in the preterm infant group begins to reflect the levels of the full-term infants.

Nephrogenesis, completed in humans by ~36 wk (2), is a sequence of changes that involves the interaction of mesenchyme and ureteric bud transitioning into a complex and highly regulated nephron (15). The process is controlled in part by several families of growth factors that interact with extracellular matrix proteins and cell-surface receptors (16). If all of these processes occur as programmed, the kidney develops a complement of nephrons adequate to maintain homeostasis in a wide range of environmental stresses throughout life. Therefore, it was expected that various growth factors would be identified in the urine of preterm infants still completing nephrogenesis.

Our results showing elevated levels of multiple members of the IGF family support the critical role for this family of growth factors during nephrogenesis. Animal studies have shown that transgenic overexpression of IGFBP-1 correlates with a reduction of nephrons as mice pups age. This reduction is presumed to be because of a reduction of IGF bioavailability (17). IGF-II has been shown to augment nephrogenesis in organ culture

(17). The significance of our finding of higher IGFBP-1 in the urine of preterm infants at birth is unclear. It is known that the IGFBPs have specific roles during nephrogenesis to modulate the actions of IGF regulating mesenchymal induction, differentiation, and growth (18). Transient overexpression of IGFBP-1 may be pathologic and result in a diminished nephron number. Alternatively, increased IGFBP-1 may merely be a mechanism for decreasing the bioavailability of IGF in the kidney, signaling the completion of nephrogenesis.

Although the identification of growth factors in the urine of preterm infants was expected, the other markers identified in this study (MCP-1, CD14, and possibly Siglec-5) are often considered markers of “inflammation” and were unanticipated findings. No other studies have previously identified these proteins in the urine of preterm human infants, and there is limited information about these inflammatory markers during renal injury. We propose two plausible reasons for their existence in the urine of preterm infants: direct role in normal renal development or reflection of a systemic state of stress.

MCP-1 is a chemokine that attracts monocytes and is elevated in patients with ureteropelvic junction obstruction (19) and renal transplant rejection (20). CD14 is expressed on many cells and has been found to be elevated in the blood of patients with chronic renal disease, possibly as a marker for increased risk of death in patients on dialysis (21). Of note, CD14 and MCP-1 may interact to create fibrosis (22). In a study focusing on keloid formation, enhanced MCP-1 release by CD14+ cells augmented fibroblast proliferation (23). We hypothesize that increased urinary excretion of both MCP-1 and CD14 in preterm infants may reflect increased risk of early renal fibrosis and its long-term consequences. Although both *MCP-1* and *CD14* genes are expressed in the developing murine kidney (24), their function within the developing kidney has not been established. Siglec-5 is a large cell-surface receptor (61 kDa) and member of the immunoglobulin superfamily (25). Renal expression of Siglec-5 and its potential function in renal development is entirely unknown. There is no gene expression data regarding Siglec-5 in the GUDMAP database (24).

It is important to consider the possibility that the inflammatory markers found in preterm infants may not be directly related to renal development. Preterm delivery is often the consequence of a stressful maternal environment. Maternal conditions such as preeclampsia, hypertension, or infections may result in systemic inflammatory responses in the infant with a subsequent increase in the urinary excretion of those markers.

It is intriguing that the differences between the preterm and full-term infants persisted for as long as 6 mo. Preterm IGFBP-6, Siglec-5, and CD14 levels were equivalent to term levels by 2 mo, whereas IGFBP-1, IGFBP-2, and MCP-1 levels were equivalent by 6 mo of age. The cohort of preterm infants ($n = 13$) was at most 4 wk from full term (33 wk, $n = 4$) and included infants only 2 wk from full term (35 wk, $n = 3$). If indeed these markers represent the presence of ongoing renal development, it is possible that preterm infants may have an extended period of renal development (normal and/or abnormal) after birth. Alternatively, early

after parturition, signaling pathways may be initiated to adapt to an *ex utero* environment. In either case, it is conceivable that inflammatory markers such as those identified in this study may eventually be used to identify infants at risk of chronic kidney disease in adulthood.

Preterm infants are vulnerable to numerous pathologic processes, including the alteration of nephrogenesis by intrauterine and postnatal environmental stressors (4,5). These influences could result in long-term modifications in renal function and structure undetectable with current methods of clinical evaluation, but ultimately culminating in the known 70% increased risk of chronic kidney disease in adults whose birth weight was <2.5 kg (11). As the number and cost of infants born prematurely and adults with chronic kidney increase, identification of infants at risk is imperative. Education regarding prevention of further renal damage and avoidance of nephrotoxic drugs could play an important protective role if high-risk infants could be identified before manifestations of adulthood disease. Likewise, identification of biomarkers that predict the eventual development of adult disease may indicate potential therapeutic targets that could result in improved long-term renal outcomes. This study reveals marked differences in urinary cytokines between infants undergoing completion of nephrogenesis and those undergoing normal postnatal renal maturation. Gestational and postnatal age affect the urinary levels of many proteins and cytokines (26). Another study has examined the role of gestational age within the urinary proteome looking at pre-selected markers. Urinary levels of neutrophil gelatinase-associated lipocalin, osteopontin, and beta-2 microglobulin decreased as the gestational age of the infant increased (26). Our study used a targeted approach to identifying markers of renal development and we have discovered a number of novel markers that may play a role in renal development or renal injury. To confirm these markers as useful biomarkers of renal development, they must be correlated to histologic stages of kidney development and confirmed as mechanistically important. As the search for biomarkers of renal injury progresses, gestational, and postnatal ages are critical factors in the evaluation of the validity of a urinary biomarker as a surrogate for renal disease. As surrogates of renal development are discovered, clues to how nephrogenesis can be augmented or protected may emerge.

In conclusion, the cell-surface receptors, soluble ligands, transcription factors, growth factors, and structural proteins essential for nephrogenesis are logical potential biomarkers of normal and abnormal renal development. The variations seen in the markers identified in this project may represent changes in normal developmental processes or adaptations to the extra-uterine environment that are related to preterm delivery. Studies of preterm infants at earlier gestational ages may identify additional indicators of earlier phases of nephrogenesis, but are also expected to be complicated by the effects of the significant comorbidities associated with extremely low birth weight. These results support the previous indications that, when evaluating the utility of a urinary biomarker in children, both gestational and postnatal age must be considered (S.C.K., personal communication).

Ongoing work to identify useful biomarkers of renal health and disease in childhood could ultimately result in improved renal health and survival throughout life.

METHODS

Subject Selection

The project was approved by the University of Virginia Institutional Review Board and informed consent was obtained for each patient. Patients were recruited from the newborn nursery and NICU at the University of Virginia Children's Hospital. Infants were enrolled in two groups: preterm (33–35 wk post-conceptual age) and term (38–40 wk post-conceptual age). A physical exam, including estimation of gestational age by Ballard exam, demographics, and prenatal history were performed or collected from the chart. Subjects were excluded if a congenital abnormality was detected or if diagnosed with a urinary tract infection.

Urine Collection

Urine was collected within 48 h of birth using a U-bag collection device. The urine was centrifuged, aliquoted and stored in a -80°C freezer for analysis of protein, creatinine, and cytokines. The clinical status of the infants was evaluated and urine samples were collected at designated time points (Figure 2).

Initial Identification of Urine Biomarkers

Eight preterm and eight full-term infant urine samples were analyzed at birth and 6 mo using a G-2000 antibody array kit (RayBioTech, Norcross, GA). The array detects 174 cytokines, mainly growth factors, adhesion molecules, and soluble receptors using antibodies embedded on a glass slide in a gridded pattern (complete list in **Supplementary Material**, online). This array was chosen as it was the largest commercially available kit for screening logical candidate markers of renal development. The urine sample was applied undiluted and the proteins were detected in duplicate by fluorescence labeling. An Agilent laser scanner (Agilent Technologies, Santa Clara, CA) was used to detect the relative fluorescence and Feature Extraction software (version 9.1.3, Agilent Technologies) converted the data to numerical form. The RFI was normalized to the positive control within the array and to the sample urine creatinine concentration (mg/dl). The DetectX Creatinine Urinary Detection Kit (Arbor Assays, Ann Arbor, MI) was used to measure urine creatinine by a modified Jaffe reaction.

Selection of candidate biomarkers. The search for candidate markers was initially on the basis of the degree of RFI normalized for sample creatinine. The cytokines were stratified into four groups: RFI $>1,000$, 500–1,000, 200–500, and <200 . A protein was assigned to a group on the basis of the highest measurement in any of the four groups assessed (preterm–birth, preterm–6 mo, term–birth, and term–6 mo). Cytokines with an RFI <200 were not felt to be quantifiable on validation studies and were not further analyzed. A cytokine was considered a “candidate marker” if the level in any group was $>1,000$ or if the cytokine levels were uniformly

higher in the preterm group at birth as compared with the term group at birth but did not reach the $>1,000$ cutoff. When reviewing the initial arrays there were no markers that appeared to be uniformly elevated in the term group at birth as compared with the preterm group.

Quantitation Studies. A customized multiplex enzyme-linked immunosorbent assay (Ray BioTech, Norcross, GA) was designed for quantification of a selected group of proteins identified in the discovery arrays and analyzed in quadruplicate. These proteins consisted of IGF-I, IGF-II, IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5, IGFBP-6, MCP-1, CD14, and Siglec-5. The IGF protein family (IGF-I, IGF-II, and IGFBP-1–6) was chosen for further analysis as both IGFBP-1 and IGF-II were elevated in preterm infants at birth as compared with the term infants at birth. MCP-1, CD14, and Siglec-5 were selected as they were consistently elevated in the preterm group at birth. MCP-1 and CD14 were selected for further evaluation as they have a role in renal development (24) and disease (19,21). Siglec-5 has no known role in renal development but it is large, unlikely to be filtered by the nephron, and could represent a unique, previously unexamined marker for renal development.

To confirm the semi-quantitative differences seen in the initial screening analysis, quantification studies were performed on the same samples used in the initial arrays (birth and 6-mo urine samples) with the addition of the other urine samples collected up to 12 mo (Figure 2). Thirteen infants in each group completed urine collections through 6 mo. Of those 13 infants, 8 in the term group and 7 in the preterm group contributed a urine sample at 12 mo of age.

Statistical Analysis

Initial array: candidate markers. Sigma Stat software was used to perform nonparametric tests as data were not normally distributed. The preterm group was compared with the term group at two time points: birth and 6 mo using the Wilcoxon signed-rank test. Both the preterm and term groups were compared within each group over time (birth as compared with 6 mo) using the Mann–Whitney rank sum test.

Quantification studies. Linear mixed models that included a random intercept and slope and allowed for an auto-regressive residual correlation were used to compare changes in biomarkers over time within the term and preterm groups. The fixed effects included to make these inferences included a quadratic function of time (centered to avoid collinearity), separate for each group. Biomarker levels were analyzed in the log-scale to better match the assumptions underlying the models. F-tests on the basis of contrasts were used to compare the preterm and term groups at specific time points. The Kenward–Roger approximation to the degrees of freedom for the associated tests was used to ensure accurate inference for the small sample size. Analyses were carried out using SAS 9.2.2 PROC MIXED.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/pr>

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Figure 2. Timeline for urine collections in the preterm and full-term infants. The timeline for urine collections is based on postconceptional age in preterm infants from birth until 6 mo of age. The collection due at 1 y was collected on the infant's date of birth.

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