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Human Nephrogenesis can Persist Beyond 40 Postnatal Days in Preterm Infants

James Carpenter[1], Sunitha Yarlagadda[1], Katherine A. VandenHeuvel[2,3,4], Lili Ding[3,5] and Meredith P. Schuh[1,3,6]

[1]Division of Nephrology and Hypertension, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA; [2]Division of Pathology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA; [3]Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA; [4]Department of Pathology and Laboratory Medicine, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA; [5]Division of Biostatistics and Epidemiology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA; and [6]Division of Developmental Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA

Introduction: Human nephrogenesis is typically completed by 36 weeks gestation; however, it is impacted by preterm birth. Early studies suggested that nephrogenesis persisted for ≤40 postnatal days in preterm infants. However, the postmenstrual age (PMA) of the preterm infants who survived >40 days was uncertain. In this study, we sought to reexamine postnatal kidney development in preterm infants surviving >40 days.

Methods: Human kidney samples were obtained from an institutional biobank. Samples were considered controls if survival was ≤4 days after birth with PMA of 30 to ≤36 weeks. Kidneys from preterm neonates with postnatal survival >40 days and PMA of 30 to ≤36 weeks were compared to controls. We counted glomerular generations, measured nephrogenic zone widths (NZW), and performed immunofluorescence (IF) with SIX1 and RET. We compared kidney weights and quantified the cross-sectional area of proximal (lotus tetragonolobus lectin [LTL], SL22A2), distal (SLC12A3, KCNJ10), and glomerular (nephrin) markers using IF.

Results: Seven preterm infants surviving >40 days and 8 controls were analyzed. Four of 7 preterm infants had histologic and molecular evidence of nephrogenesis. Cessation of nephrogenesis in preterm infants occurred 2 weeks earlier than PMA-matched controls with attenuated expression of both SIX1 and RET. We found increased kidney weight-to-body weight ratio, increased distal tubular cross-sectional staining in the superficial nephrons, and distal tubular hypertrophy and hyperplasia in the preterm infant kidneys.

Conclusion: Our study supports that nephrogenesis in preterm infants persists longer than previously thought with evidence of early nephron stress, placing importance on the neonatal environment.

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Human nephrogenesis is complete prior to birth at 34 to 36 weeks gestation, with 60% of nephrons forming during the third trimester.[1] Preterm birth is associated with low nephron endowment, with an increased risk of chronic kidney disease later in life.[2-4] The reasons for this low nephron number are unclear; however, the current assumption is that nephrogenesis persists for 40 days or less postnatally based on previous autopsy studies.[5] These early studies are based on histologic definitions of nephrogenesis and reflect best practice at the time. Importantly, the previous literature left some uncertainty about the PMA of the preterm infants analyzed who survived more than 40 days, because the mean PMA of the infants studied (without kidney failure) was 63 weeks. This PMA is after the period of typical nephrogenesis of full-term infants. It is unclear how many samples were included that survived >40 days with a PMA prior to the typical 34 to 36 week nephrogenesis cessation timing. In order to understand the previous literature and the goals of our study, it is important to define common terms surrounding pregnancy and preterm birth. Gestational age (GA) is clinically defined as the time (weeks) that elapsed from the first day of the last

Correspondence: Meredith P. Schuh, 3333 Burnet Avenue, MLC 7022 Cincinnati, Ohio 45229, USA. E-mail: Meredith.schuh@cchmc.org

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menstrual period until the day of delivery[6,7] (Supplementary Figure S1). Chronological age (CA) is the time (days, weeks, months, and years) that elapsed since birth. PMA (PMA, weeks) is the GA plus the CA. Therefore, an infant born at 24 weeks gestation who survived 8 chronological weeks would have the same PMA as an infant just born at 32 weeks gestational age (wga). As the survival of those born extremely preterm (<28 wga) continues to improve, demystifying the process of postnatal nephrogenesis becomes increasingly important to long-term kidney health.

In this study, we sought to reexamine postnatal development with modern technology on a cohort of infants with PMA limited to 30 to 36 weeks. We focused on the window of 30 to 36 weeks PMA to capture both ongoing nephrogenesis and the typical window of nephrogenesis cessation. On histologic examination, we found that some, but not all, preterm infants continue nephrogenesis postnatally, for up to 62 days, longer than is conventionally believed. We identified a trend toward earlier cessation in extremely premature infants by 2 weeks compared to PMA-matched controls, although still within the window of typical nephrogenesis cessation. Furthermore, we corroborated previous findings of increased kidney weight-to-body weight ratio in preterm infants, with distal tubular hypertrophy and hyperplasia prior to proximal tubular changes in the more recently formed nephrons. Our study supports that nephrogenesis persists longer than previously thought in the preterm infant with evidence of early nephron stress, placing even more importance on the neonatal environment and early exposures.

METHODS

Patient Selection and Kidney Samples

All human kidney samples were obtained from the Cincinnati Children's Hospital Medical Center Biobank after review and approval by the internal review board for use in the Discover Together Biobank. The Discover Together Biobank is a resource that facilitates the acquisition, processing, storage, and distribution of biospecimens for research studies. Consent for storage and future use of biobank tissue was obtained at the time of the autopsy. Tissue for biobank storage was fixed in formalin and preserved as formalin-fixed paraffin-embedded blocks for long-term storage. GA was determined by the obstetric or neonatologist documentation taken at the time of the autopsy. Kidneys were chosen based on GA, low maceration grade (the process of tissue autolysis[8]), and normal gross examination of the kidneys as documented in the autopsy report. Exclusion criteria included history of intrauterine growth restriction, multiparity, cystic kidney disease, chromosomal abnormality, and postmortem interval (time from death to autopsy) >96 hours. Kidneys were considered control samples if death was likely to have been intrapartum, with minimal retention time prior to delivery, or survival was ≤4 days after birth with PMA of 30 to ≤36 weeks. Kidneys from a cohort of preterm neonates with postnatal survival >40 days and PMA of 30 to <36 weeks were compared to the controls. The number of samples used was based on the sum of all available samples that met inclusion criteria for this study. Patient demographics included sex (defined by anatomy at birth/autopsy), GA, PMA, CA, birth weight, weight at autopsy, body length, and kidney weight. In addition, neonatal exposures or events including antenatal steroids, bronchopulmonary dysplasia (BPD), necrotizing enterocolitis, nonsteroidal anti-inflammatory drugs, aminoglycosides, and acute kidney injury (AKI) were assessed in the autopsy report. A composite scoring system for exposures or conditions was created. One point was awarded for antenatal steroids, BPD, necrotizing enterocolitis, aminoglycoside exposure, nonsteroidal anti-inflammatory drugs exposure, and AKI.

Tissue Preparation

Hematoxylin and eosin-stained sections of kidney tissue cut at 4 microns thickness were evaluated for width of nephrogenic zone and the number of glomerular generations, as described below. During analyses, researchers were blinded to PMA.

Glomerular Generation Number

For the initial analysis of kidney development, we used glomerular ray formation along a medullary ray from the cortico-medullary junction to the outer kidney cortex as previously published.[5,9,10] Medullary rays were determined to be a series of mature glomeruli along a linear path extending from the kidney cortex to the cortico-medullary junction. An average number of medullary rays from 4 distinct regions per kidney were chosen. In settings where the medullary ray was difficult to define, a line of best fit was determined by drawing a straight line from the cortico-medullary junction to the peripheral cortex and counting all mature glomeruli along that line.

Nephrogenic Zone Width (NZW)

The width of the nephrogenic zone was determined by the area in the outer kidney cortex exhibiting developing nephrons in the form of s-shaped or comma-shaped bodies.[9,10] Four distinct regions per kidney were chosen. The average width was calculated digitally using the Elements 5.3.0 annotation tool measuring both area and length of total region.

Assessment of Glomerular Cross-Sectional Area

Immunofluorescent staining (IF) was performed as previously described[11] using guinea-pig antinephrin (Progen Biotechnik, GP-N2, Heidelberg, Germany) to quantify the number and area of glomeruli. NIS Elements was used to identify glomeruli using artificial intelligence technology trained to recognize manual segmentation, antinephrin staining in the 750-excitation channel, and DAPI staining. We counted the number of glomeruli in 5 fields of view, each 2000×2000 pixels (1.658 mm^2), and determined the cross-sectional area per glomeruli. To avoid regional bias of the section, quantification of antinephrin cross-sectional area was performed on 2 distinct sections at least 30 μm from the previous section. Because of poor IF staining, case 9 (31 wga control), case 14 (35.4 wga control), and case 15 (36 wga control) were excluded from this portion of the analysis.

Immunofluorescent Assessment of Nephrogenesis

IF staining was performed using antibodies to rabbit anti-SIX1 (cell signaling technology, 12891S, Danvers, MA), rabbit anti-SIX2 (Proteintech, 115621AP, Rosemont, IL), rabbit anti-WT1 (Abcam, ab202639, Cambridge, United Kingdom), goat anti-RET (R&D Systems, AF1485, Minneapolis, MN), guinea pig antikeratin K8 (PROGEN Biotechnik, GP-K8), and mouse anti-E-cadherin (BD Biosciences, 610182, Franklin Lakes, NJ). Images were acquired on the Nikon Ti2 Inverted SpectraX at 10× and 20× objectives. Tile scan images were obtained, and the cortical perimeter was measured using Elements 5.3.0 annotation tools. The SIX1+ niches and RET+ tips were manually counted and divided per 1 mm of cortical distance of the entire cortical perimeter. To avoid regional bias of the section, quantification of SIX1 and RET per cortical distance was performed on 2 distinct sections at least 30 μm from the previous section.

Quantification of Proximal and Distal Tubules

IF staining was performed using rabbit anti-SLC12A3 (Sigma-Aldrich, HPA028748, St. Louis, MO) and rabbit anti-KCNJ10 (Alamone, APC-035, Jerusalem, Israel) to detect distal tubules and biotinylated LTL (Vector Laboratories, B-1325-2) and SLC22A2 (Abcam, ab170871) to detect proximal tubules. DAPI was used to detect all nuclei. Tile scan images were acquired on the Nikon Ti2 Inverted SpectraX at 10x objective of the entire kidney section. To standardize differences in the cortical area based on tissue block differences, we selected 5 distinct regions of the cortical edge of 2000×2000 pixels (1.658 mm^2) and determined the average area of proximal and distal tubules per region using NIS Elements 5.3.0. This method was repeated along the cortical-medullary border, as well as the center of the cortex (Supplementary Figure S2). To avoid bias with a square-shaped region, we also defined the region of interest in the center of the cortex using a circle with a cross-sectional area of 1.227 mm^2. The cross-sectional area of the staining was averaged between the 5 regions of interest per region (cortical border, medullary border, and middle of cortex). To avoid regional bias of the section, quantification of LTL and SLC12A3 cross-sectional area was performed on 2 distinct sections at least 30 μm from the previous section. We developed a binary mask (delineation of region of interest based on signal) using DAPI to count tubular nuclei associated with SLC12A3, KCNJ10, LTL, and SLC22A2, respectively. The average number of tubular nuclei per stain within the 5 regions were also quantified. Given the limited amount of tissue available, the quantification for KCNJ10 and SLC22A2 was only performed on 1 section. Due to concern for autolysis and poor tubular staining on case 9 (31 wga control) and case 15 (36 wga control), these samples were excluded from this portion of the analysis. In addition, case 14 (35.4 wga control) was not used for nuclear analysis due to lack of DAPI staining despite adequate tubular staining.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism v9 (La Jolla, CA) and R-4.3.1. Mean ± SD was determined for the NZW and glomerular generation number. Linear regression was used to determine the correlation of fetal indices and GAs. Nonpaired *t*-test or Mann-Whitney (for nonnormally distributed data) was used to determine statistical significance between controls and preterm infants for SIX1+ and RET+ per mm of cortical distance, LTL, SLC22A2, SLC12A3, KCNJ10 staining, and nuclear counts.

RESULTS

The experimental cohort was comprised of 7 preterm infants surviving >40 chronological days (Table 1) and 8 infants with a PMA of 30 to 36 weeks surviving <4 days serving as controls. There was no significant difference in PMA, PMI, autopsy weight, kidney weight, or infant length by either crown-heel or crown-rump length between preterm infants and controls. The timing of nephrogenesis cessation and cause of death are summarized in Supplementary Figure S3.

Morphologic Assessments of the Nephrogenic Niche Show Persistent Nephrogenesis Past 40 Days in Some but Cessation Occurring Before PMA-Matched Controls

We performed glomerular generation counts[5,9,10] (Supplementary Figure S4) and observed no

Table 1. Sample characteristics of postmenstrual controls and preterm neonates

Characteristics	Control ($n = 8$)	Preterm ($n = 7$)
Gestational age (weeks)	33.01 ± 2.34 (30.00–36.00)	25.41 ± 1.51[a] (23.90–28.00)
Postmenstrual age (weeks)	33.17 ± 2.25 (30.57–36.00)	32.27 ± 1.46 (30.1–34.00)
Chronological age (days)	1.00 ±1.51 (0–4)	49.71 ± 8.24[a] (41–62)
Male (%)	25	28.5
Postmortem interval (hours)	40.25 ± 29.63 (10–96)	32.29 ± 29.28 (14–96)
Birth weight (g)	1817.75 ± 420.61 (1022–2235)	760.80 ± 152.59[a] (564–900)
Autopsy weight (g)	1809 ± 425.84 (1022–2235)	1489 ± 341.09 (910–1845)
Kidney weight (g)	15.83 ± 7.07 (5.90–28.90	21.29 ± 3.47 (16.10–25.70)
Crown-heel length (cm)	39.22 ± 8.49[b] (24.70–45.40)	39.33 ± 3.41 (34.40–44.40)
Crown-rump length (cm)	29.11 ± 4.66[b] (21.50–33.00)	29.44 ± 1.40 (27.00–31.20)

[a] $P < 0.05$ compared with control group.
[b] $n = 5$ for control group.

statistically significant difference in the glomerular generations of controls versus preterm infants surviving >40 days, supporting comparable nephron numbers between these 2 groups (Figure 1a and b). The average NZW was 50.33 ± 55.55 μm in the preterm infants compared to 77.21 ± 54.43 μm in PMA-matched controls (Figure 1c). Further evaluation investigating differences in NZW by PMA revealed a significant correlation between increasing PMA and decreasing NZW. Both the preterm and controls showed a significant correlation between increasing PMA and decreasing NZW, but with a striking shift in the x-intercept, with the preterm infants intersecting at 34.21 weeks and controls at 36.88 weeks (Figure 1d), indicating cessation 2 weeks earlier in the preterm infants than the PMA-matched controls. Based on the histologic assessment, only 1 experimental sample had no evidence of a nephrogenic zone (Supplementary Figure S3, case 5: PMA 33 weeks, CA 46 days), whereas 2 others were nearing completion or almost at completion (case 4: 23.1μm, PMA 32 weeks, CA 46 days; and case 7: NZW of 1.16μm, PMA 34 weeks, CA 41 days). In the control group, 2 samples had completed nephrogenesis (case 13: PMA 35.2 weeks, CA 2 days; and case 14: PMA 36 weeks, CA 0 days). Examples of NZW can be seen in Figure 1e.

Molecular Assessments of the Nephrogenic Niche Demonstrate Ongoing Markers of Nephrogenesis but Reduced Expression of SIX1 and RET in Preterm Infants From 30 to 36 Weeks PMA Relative to Controls

To determine molecular timing of nephrogenesis cessation, we performed IF staining of the nephrogenic niche. Nephron formation requires inductive signals between the nephron progenitor cells in the cap mesenchyme and the ureteric tip.[12] Using known nephron progenitor cell markers SIX1, SIX2, and WT1, and ureteric tip marker RET, we assessed for any molecular evidence of ongoing nephrogenesis in the preterm group. Notably, 4 of the 7 preterm samples showed evidence of ongoing positive SIX1 and RET staining (Figure 2a and b). We next stained for SIX2 and WT1 in the preterm samples (Supplementary Figure S5). Most samples contained WT1+ signal in the nephrogenic niche, but minimal SIX2 staining, supporting the previous literature that SIX1 may be maintained longer than SIX2 in the human kidney.[13] In addition to the nephrogenic niche, SIX1 is also expressed in the nascent nephron (renal vesicle, s-shaped body).[14] Because SIX1 is expressed both in the nephron progenitor cells and nascent nephron whereas RET is only expressed in the ureteric tip during active nephrogenesis, we relied on RET+ signal as the most accurate surrogate for active nephrogenesis.[15] In addition, we stained for e-cadherin as a marker of distal tubular epithelialization and commitment to differentiation.[11] To identify if the preterm samples were nearing nephrogenesis cessation earlier than their PMA counterparts, we counted the total number of SIX1+ regions and RET+ niches along the entire perimeter of the cortex. There was a trend toward decreased SIX1+ regions (Figure 2c, $P = 0.117$) and RET+ active niches in the preterm kidneys (Figure 2d, $P = 0.062$) relative to controls (Figure 2d); however, the data did not allow for statistical comparison in this analysis due to sample size. We then analyzed the differences in SIX1 and RET expression by PMA using simple linear regression. As expected, we identified a linear relationship of decreasing expression with increasing PMA with the controls (Figure 2e and f; SIX1 $R^2 = 0.54$, $P = 0.038$; RET $R^2 = 0.79$, $P = 0.003$). This correlation did not hold for the preterm infants, where relatively flat lines with nonsignificant slopes are seen for both SIX1 ($P = 0.60$) and RET (0.07), emphasizing decreased expression at earlier PMAs than the PMA-matched controls. Two experimental samples displayed molecular cessation of nephrogenesis before histologic cessation (Supplementary Figure S3, cases 4 and 7). Case 5 displayed

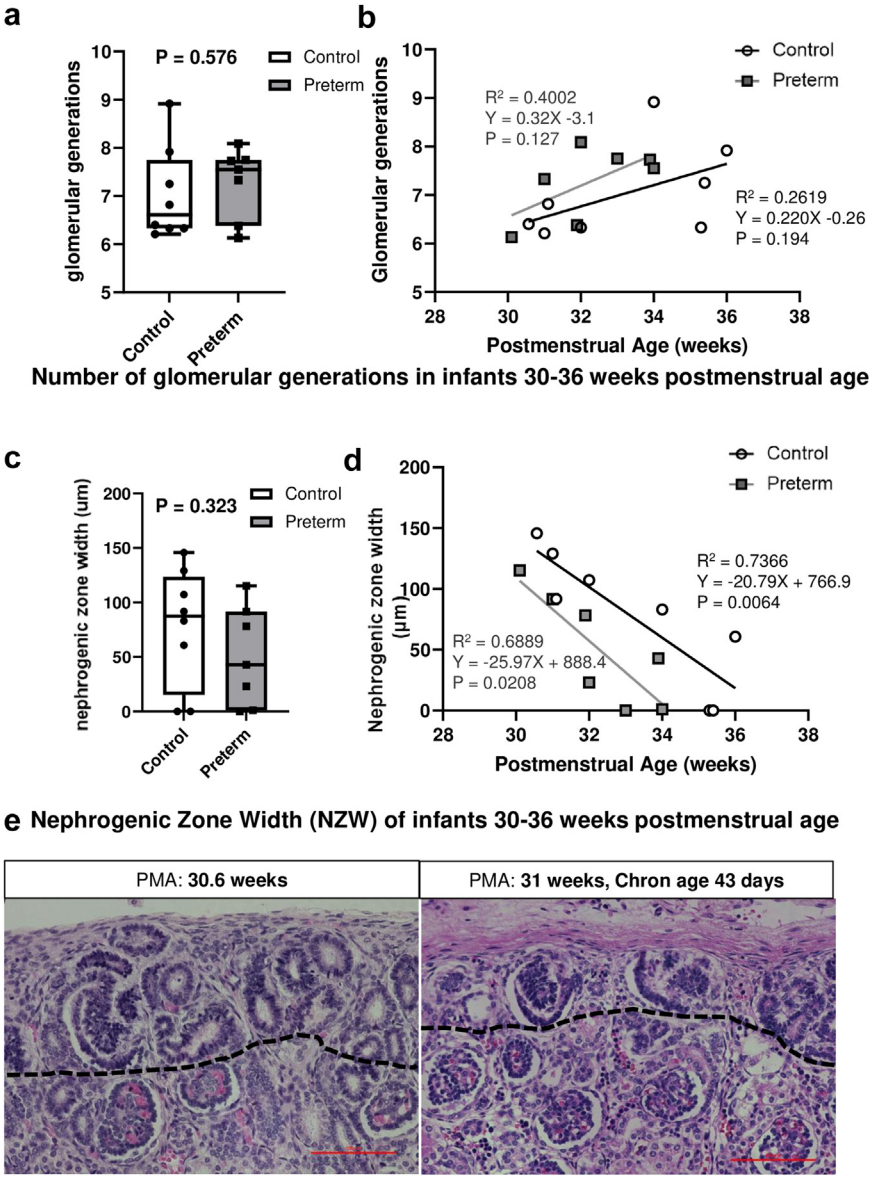

Figure 1. Morphologic assessments of the nephrogenic niche. (a) No significant differences noted in the number of glomerular generations in preterm infants surviving >40 chronological days and postmenstrual age matched controls. (b) Linear regression analysis of the number of glomerular generations formed within the kidney versus postmenstrual age. (c) No significant difference noted in the nephrogenic zone width between preterm infants surviving >40 chronological days and postmenstrual age matched controls. (d) Linear regression analysis of the number of glomerular generations formed within the kidney vs postmenstrual age shows shift toward earlier nephrogenesis cessation. (e) Examples of nephrogenic zone widths. NZW, nephrogenic zone width; PMA, postmenstrual age.

molecular and histologic cessation of nephrogenesis. The full summary of NZW, SIX1+ regions, and RET+ niches per case can be seen in Supplementary Figure S3.

Preterm Infants Surviving Greater Than 40 Chronological Days Have Enlarged Kidneys With Significant Distal Tubule Hypertrophy and Hyperplasia and Early Proximal Tubule Hypertrophy

The combined kidney weights of the preterm infants who survived >40 days trended toward being larger (Figure 3a, $P = 0.087$). After normalizing kidney weight to body weight at the time of autopsy, the kidney weights in the preterm infants became significantly larger (Figure 3b). Because of the concern that these differences in body weight may be confounded by fluid status, we normalized kidney weight to infant length, using both crown-heel and crown-rump length[16] (Figure 3c and d). The increased kidney size became even more striking when normalizing to body length, confirming our findings of larger kidneys in preterm infants. The cortical width appeared to be increased in the preterm infants relative to controls (Figure 3e); however, this may be biased to the tissue section on the pathology block and was therefore not

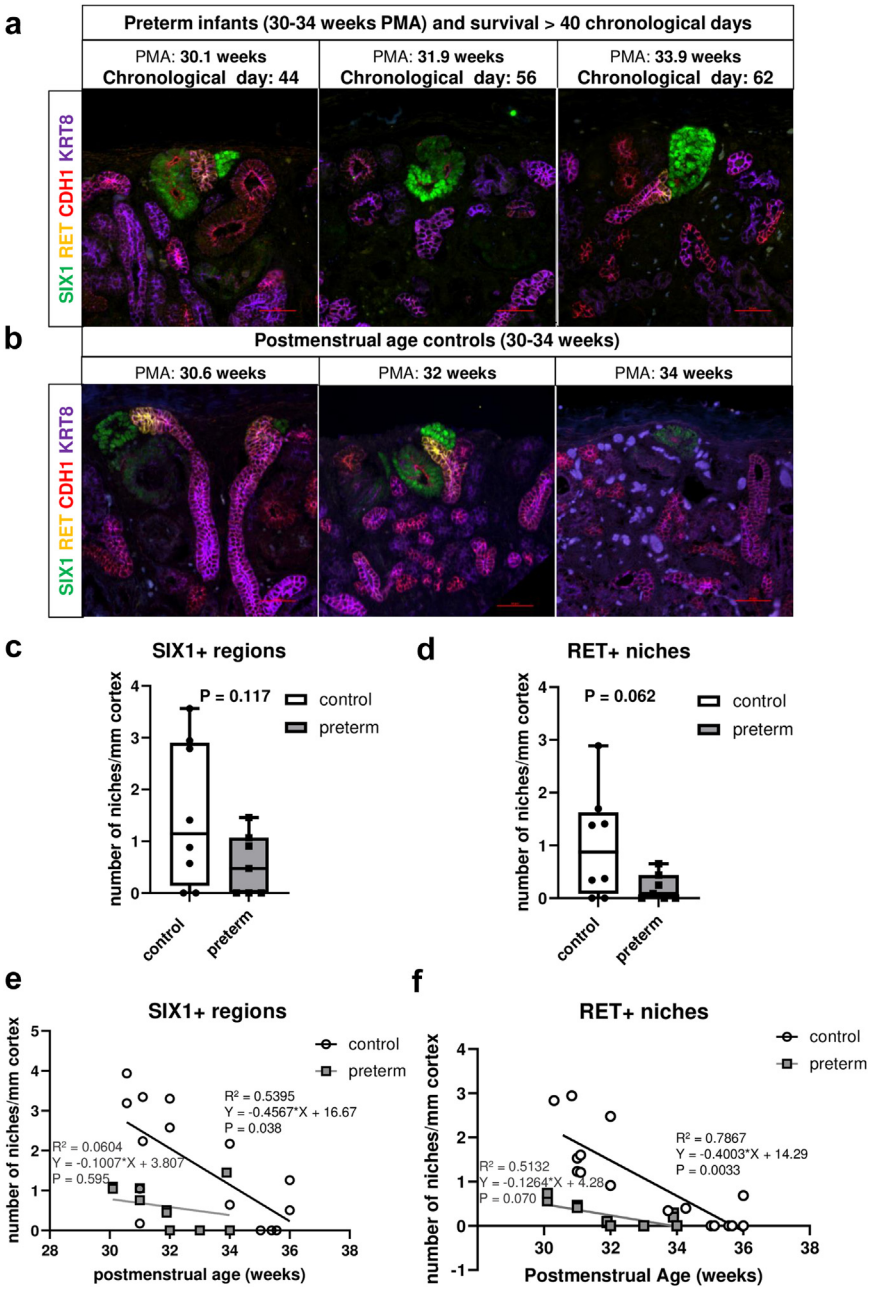

Figure 2. Molecular evidence of ongoing nephrogenesis in postnatal preterm kidneys. (a) Ongoing nephrogenesis visible by SIX1+ (green) and RET+ (yellow) staining in the preterm infants with appearance similar to PMA-matched controls (b). Although there is no significant difference, there is a trend toward overall decreased expression of SIX1+ regions (c) and RET+ niche tips (d) per mm of cortical distance in the preterm infants relative to controls. Linear regression of (e) SIX1+ regions and (f) RET+ niches per mm of cortical distance versus postmenstrual age. There is a poor correlation for both markers of nephrogenesis with loss of expression approximately 2 weeks earlier than postmenstrual age controls. PMA, postmenstrual age.

formally quantified. When comparing kidney weight to body weight and length relative to PMA, we identified a trend of decreasing kidney size with increasing PMA, suggesting that those most preterm who survive <40 days have the greatest compensation (Supplementary Figure S6).

We next sought to determine if this increase was due to tubular hyperplasia and/or hypertrophy in the postnatal period, given the data suggesting equivalent glomerular numbers. We standardized the cortical cross-sectional area of assessment between preterm infants and controls (see Methods). Assessing the cortical border, middle cortex, and medullary border, we observed no difference in LTL+ cross-sectional staining between preterm infants and controls (Figure 4a and b). However, we did identify a significant increase in SLC12A3+ cross-sectional area (Figure 4c) in all regions of the cortex and confirmed on 2 distinct slide

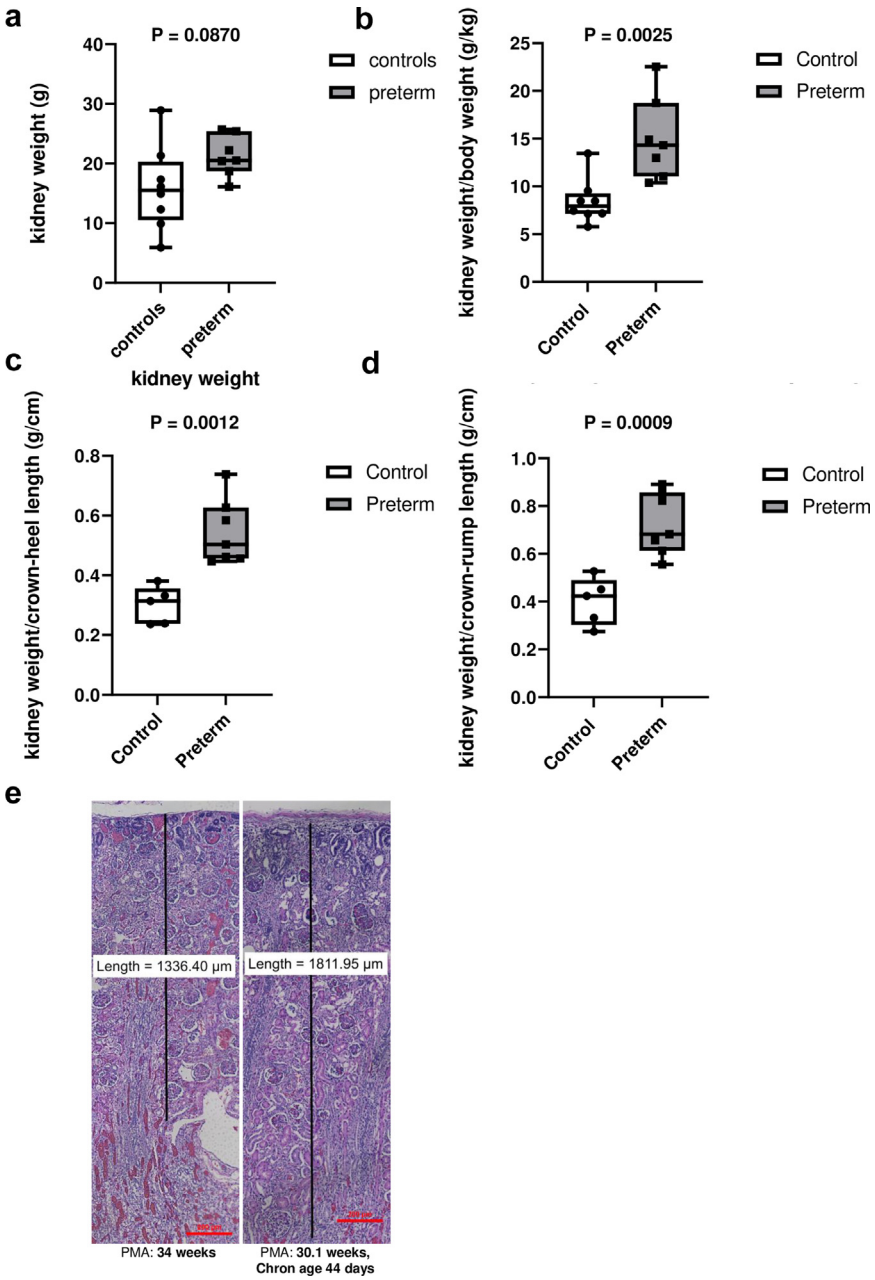

Figure 3. Preterm kidneys are larger than postmenstrual-age matched controls. (a) The combined kidney weights of preterm infants who survive >40 days are not significantly bigger than PMA-matched controls. (b) Normalizing for body weight, the preterm kidneys were significantly larger relative to PMA-matched controls. To minimize the confounding effect of variations in fluid status, the kidney weight to crown-heel length (c) and kidney weight to crown-rump length (d) were also assessed and the preterm kidneys remained significantly larger relative to controls. (e) Example of cortical width difference in preterm infants and control. PMA, postmenstrual age.

sections. We then analyzed the differences in SLC12A3+ and LTL+ expression by PMA using simple linear regression. We identified a significant linear relationship of increasing SLC12A3+ expression with increasing PMA in the preterm infants which was not found in the controls nor the LTL+ staining in either preterm or control (Supplementary Figure S7). To determine whether this finding was secondary to increased cellularity or hypertrophy, we quantified tubular nuclei adjacent to SLC12A3+ staining (Figure 4d, Supplementary Figure S8). There was no significant increase in peritubular nuclei number around SLC12A3+ tubules in the preterm infants despite the significant change in cross-sectional area supporting compensatory hypertrophy of the distal tubules in the preterm infants surviving >40 days. In fact, there were significantly fewer SL12A3+ nuclei at the medullary border in the preterm infants. To avoid bias based on the selected marker, we repeated the analysis with the distal marker KCNJ10 (Figure 5a and b). A significant

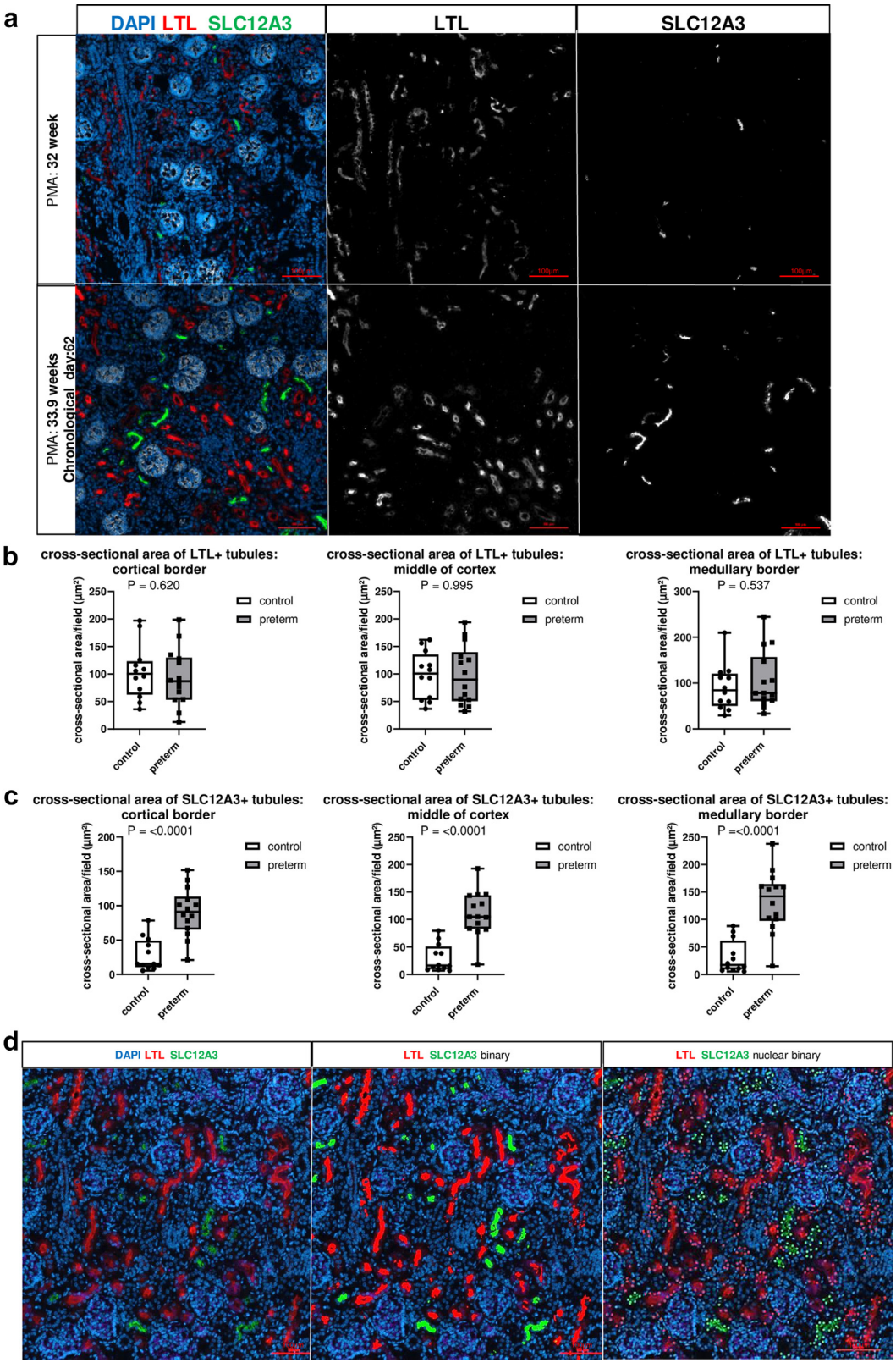

Figure 4. Preterm infants exhibit increased SLC12A3+ cross-sectional staining and distal tubular hypertrophy. (a) Example of increased SLC12A3+ cross-sectional staining (green) in preterm infants (bottom panel) relative to controls near cortical border (top panel). (b) There is no difference in cross-sectional staining of lotus tetragonolobus lectin, but (c) there is a significant increase in SLC12A3+ cross-sectional staining in all regions of the kidney. (d) Example of binary created to count cross-sectional area and nuclei.

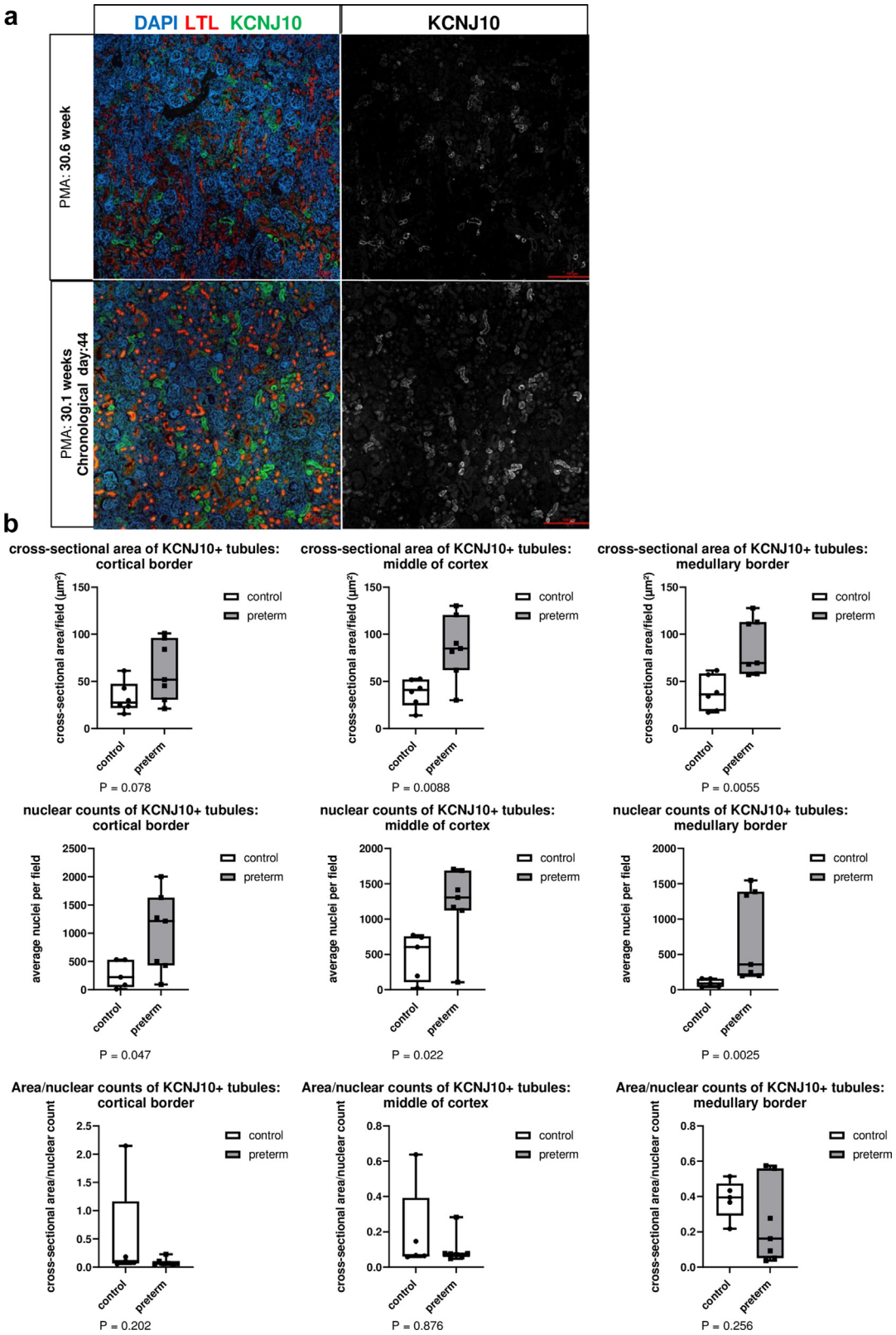

Figure 5. Preterm infants exhibit increased KCNJ10+ cross-sectional staining except at cortical border and exhibit increased cellularity in these tubules. (a) Cross-sectional staining exhibits increased KCNJ10 staining (green) in preterm infants (bottom panel) relative to controls near medullary border (top panel). (b) Increased KCNJ10 staining is seen in the middle of cortex and medullary border but not at cortical border. This increased staining is associated with increased tubular nuclear count. PMA, postmenstrual age.

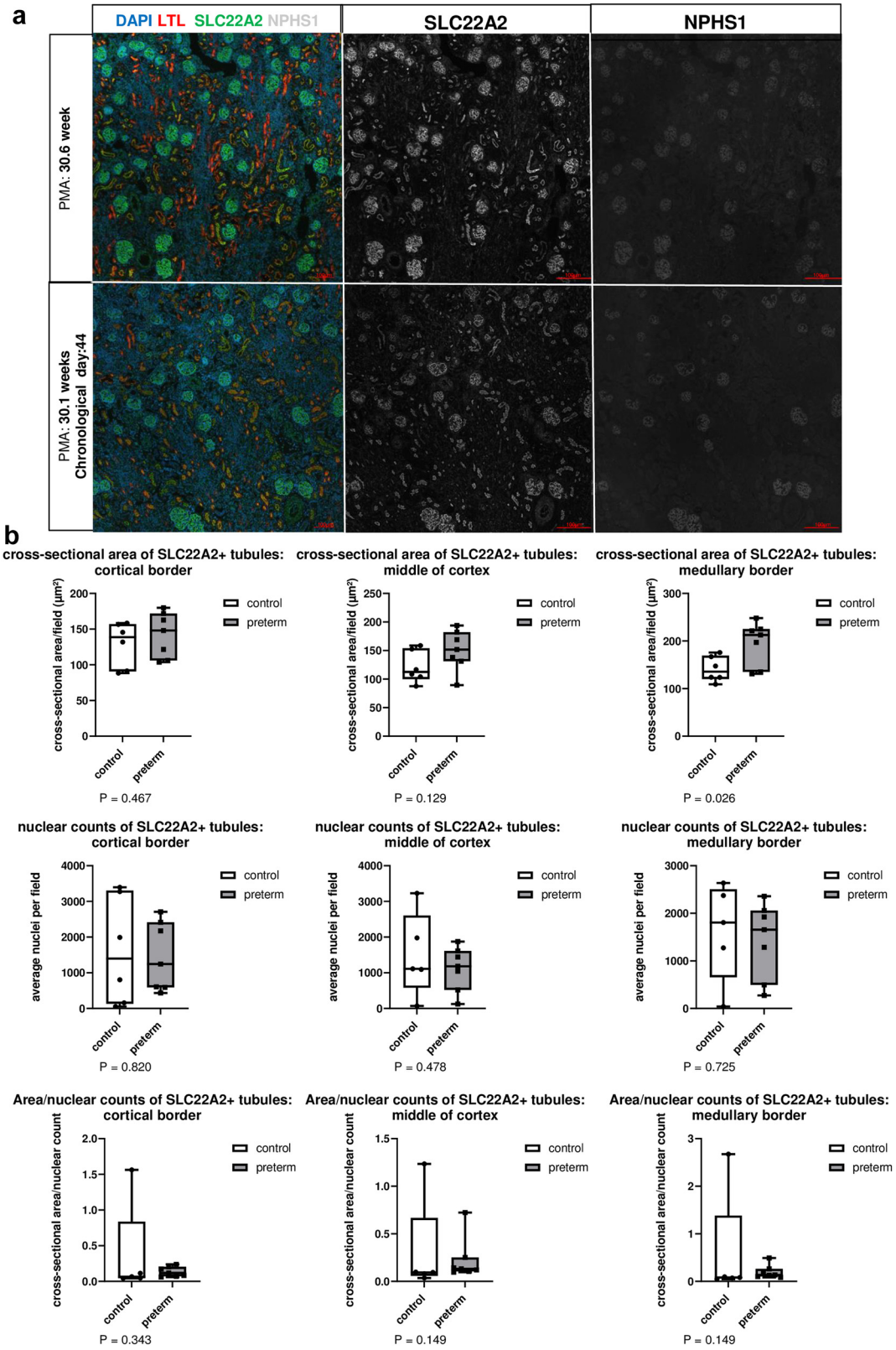

Figure 6. Preterm infants exhibit increased SLC22A2+ cross-sectional staining only at medullary border without increased cellularity in these tubules. (a) Cross-sectional staining shows SLC22A2 staining (green) in preterm infants (bottom panel) relative to controls near medullary border (top panel). Note costaining of glomeruli (NPHS1) which was subtracted for quantification of proximal tubules. (b) Increased SLC22A2 staining is seen at medullary border (deep nephrons) but not at middle or cortical border. This increased staining is not associated with increased tubular nuclear count. PMA, postmenstrual age.

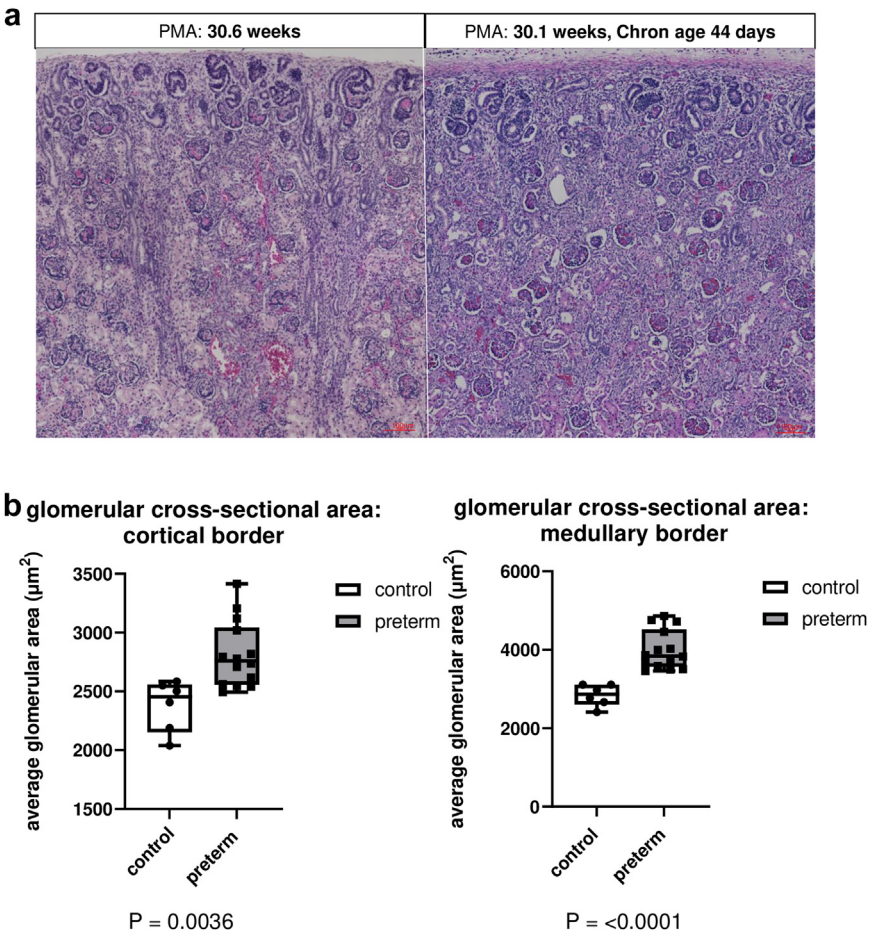

Figure 7. Preterm infants exhibit increased glomerular cross-sectional area compared to controls. (a) Example of preterm infant (left) and control (right) both exhibiting ongoing nephrogenesis (PMA = postmenstrual age). (b) Glomerular cross-sectional area was increased in both the cortical border and medullary border of the preterm infants.

increase in KCNJ10+ cross-sectional staining is seen in the middle of cortex and medullary border, but not at the cortical border. Unlike SLC12A3, there was a significant increase in tubular nuclei associated with KCNJ10 staining, suggesting hyperplasia in the distal tubules as well.

Due to concern that LTL, which binds to the carbohydrates of glycoproteins of the proximal tubles,[17] will not reflect the functional changes of the preterm proximal tubules, we additionally stained for SLC22A2, an organic cation transporter of the proximal tubule (Figure 6). SLC22A2 stained both glomeruli and proximal tubules; however, quantification was limited to proximal tubules only (see Methods) based on overlapping nephrin staining (Figure 6a). No significant difference was observed in the proximal tubular staining of the outer cortex or middle of cortex; however, increased staining was found at the medullary border ($P = 0.026$), with nuclear counts supporting hypertrophy over hyperplasia (Figure 6b). Taken together, although increases in both proximal and distal nephron cross-sectional area are noted in the deep or older nephrons, the more superficial or younger nephrons above the medullary border exhibit only distal tubular changes.

Histologic Assessment of Glomeruli Demonstrates Increased Cross-Sectional Area of Glomeruli in Preterm Infants Surviving Greater Than 40 Chronological Days Compared to Controls

Employing the same regions of interest, we evaluated the cortical border and medullary border glomerular cross-sectional area between preterm infants and controls (Figure 7). A significant increase in glomerular cross-sectional area was found at the cortical border in preterm infants (2801 ± 267.8 μm) compared to controls ((2316 ± 244.7 μm), $P = 0.028$). This increase was even more striking in the more mature or deep glomeruli. At the medullary border, the average cross-sectional area was 4059 ± 539.5 μm, 42% larger than the controls ([2850 ± 234.7 μm], $P = 007$).

Table 2. Perinatal exposures

Case	Sex	NZW (μm)	SIX1+/mm	RET+/mm	Cause of death	ANS	BPD	AKI	NEC	NSAID	AG	Composite score
1	M	115.32	1.07	0.65	respiratory failure	yes	yes	not listed	not listed	not listed	not listed	2
2	F	91.57	0.91	0.44	sepsis	yes	yes	not listed	yes	not listed	not listed	3
3	F	78.18	0.48	0.09	respiratory failure	not listed	yes	not listed	yes	yes	not listed	3
4	F	23.1	0.00	0.00	respiratory failure	yes	yes (mild)	not listed	yes	yes	yes	5
5	M	0	0.00	0.00	respiratory failure	not listed	yes	not listed	not listed	not listed	not listed	1
6	F	42.95	1.46	0.24	respiratory failure	yes	yes	not listed	not listed	Not listed	not listed	1
7	F	1.16	0.00	0.00	sepsis	not listed	yes	not listed	not listed	not listed	yes	2

AG, aminoglycoside; AKI, acute kidney injury; ANS, antenatal steroids; BPD, bronchopulmonary dysplasia; F, female; M, male; NEC, necrotizing enterocolitis; NSAID, nonsteroidal anti-inflammatory drugs; NZW, nephrogenic zone widths.
A composite scoring system for exposures/conditions was created. One point was awarded for antenatal steroids (ANS), bronchopulmonary dysplasia (BPD), necrotizing enterocolitis (NEC), aminoglycoside (AG) exposure, non-steroidal anti-inflammatory drugs (NSAIDs) exposure, and AKI documented in the autopsy reports.

Autopsy Reports Revealed Most Preterm Infants had Antenatal Steroids and BPD

The autopsy reports were reviewed for the preterm samples, and a composite scoring system for exposures or conditions was created (Table 2). Of the infants analyzed in this study, 100% had documented BPD, 57% had documented antenatal steroids, 43% had necrotizing enterocolitis, 29% had nonsteroidal anti-inflammatory drugs or aminoglycosides, and none had AKI reported in the autopsy report. Notably, case 4 had the highest composite score, as well as evidence of molecular cessation of nephrogenesis.

DISCUSSION

We comprehensively examined the histologic and molecular changes of the preterm infant kidney as it continues nephrogenesis postnatally. Even with a small sample size, we demonstrate that nephrogenesis can persist beyond 40 chronological days. By avoiding infants with intrauterine growth restriction, a known cause of low nephron endowment,[18–20] we describe the period of nephrogenesis cessation by focusing on a well-characterized, typically developing cohort of preterm infants. Although the timing of cessation is still earlier than PMA-matched controls, it remains within the normal window of 32 to 36 weeks PMA. We also demonstrate both distal tubular hypertrophy and hyperplasia specific to these preterm infants with associated increased kidney weight to both body weight and length. This compensation occurs before nephrogenesis cessation.

Our study alters the paradigm of when nephrogenesis ceases in the preterm infant, prompting consideration of factors beyond early cessation as the mechanism for low nephron endowment in preterm infants. To that end, we must consider the impact of kidney insults and exposures during this vulnerable neonatal period and specifically how these factors in the neonatal intensive care unit impact the balance of decreased formation and increased loss in the eventual nephron number. AKI, which occurs in almost 50% of all preterm infants, may contribute to early cessation of nephrogenesis.[21] In addition, over 90% of all neonates aged under 28 weeks gestation are prescribed nephrotoxic agents,[22] and their impact on postnatal nephron development cannot be ignored.

It remains unclear if a 2-week earlier cessation timing as identified in this study is enough to cause a low nephron number early in life, especially when this is within the window of normal cessation timing. Are these nephrons that form postnatally more susceptible to injury and loss, or are the more mature nephrons that are formed earlier in development the target of injury due to increased demand? Although observational, our data supports that these deep nephrons are functionally stressed as evidenced by increased glomerular, proximal, and distal tubular cross-sectional area relative to controls. Importantly, the newly formed nephrons also show evidence of stress in the distal tubules.

In the absence of human data, observations from experimental models may provide additional important insights. In a study, 1-week-old rabbits with AKI caused by exposure to the nephrotoxic agents, indomethacin and gentamicin demonstrated a reduction in glomerular number at 6 weeks of age.[23] Whether this reduction in nephron number represents early cessation or loss of existing glomeruli is not known. In another study, rats exposed to indomethacin, ibuprofen, and gentamicin during the first 5 days of life had no difference in nephron number relative to control at 14 days of life (cessation of nephrogenesis in the rat), suggesting that nephrogenesis was not impacted by nephrotoxic exposure.[24] However, similar rats given indomethacin during the first 5 days of life but allowed to mature to 6 months of age had 12% fewer nephrons compared to those not exposed,[25] suggesting an increased susceptibility to nephron loss in this cohort. Preterm baboons exposed to ibuprofen had decreased NZW, but cessation timing could not be assessed because these were fetal samples with ongoing nephrogenesis.[26] Conclusions regarding timing of

nephrogenesis or final nephron number in Sutherland *et al.*'s nonhuman primate study remains speculative, although decreased nephrogenic zone is considered a surrogate of early cessation.[9]

Kidney weight-to-body weight ratio increase has previously been reported in preterm human infants, and thought to be secondary to glomerular hypertrophy.[9,27,28] Similar to our study, Sutherland *et al.* identified a significantly greater cross-sectional area of the renal corpuscle in preterm infants and increased kidney weight-to-body weight, but the distal tubules were not discussed.[9] In the baboon model, Gubhaju noted significantly increased kidney weight and volume to body weight compared to gestational-age-matched controls.[27] They also quantified a decrease in glomerular density (glomeruli/gram of kidney) in the kidney from preterm baboons compared to gestational controls suggestive of altered growth and potentially an increase in tubular mass; however, the details of the tubular changes were not discussed. Consistent with all these studies is that stress to the nephron in the neonatal period has impact early, prior to nephrogenesis cessation. Our findings suggest that evidence of hypertrophy or hyperplasia extends beyond the glomerulus into the distal tubule, potentially leading to early maladaptive compensation. Nuclear investigations in autopsy samples are challenging due to variability in tissue handling and fixation. Our data suggest that there may be more than 1 mechanism at play resulting in increased distal tubular size; however, future work focused on cell proliferation is needed to better quantify these changes.

The question that remains unanswered is why the distal tubules of the more superficial nephrons exhibit changes before the proximal tubule, which typically reabsorbs 65% of the filtered load of the mature nephron and exhibits subsequent hypertrophy in the setting of hyperfiltration and chronic kidney disease progresssion.[29,30] The physiologic changes that occur when an infant is still undergoing nephrogenesis and must adapt its existing nephrons to function postnatally is poorly understood. Unlike the proximal tubule which shows delayed reabsorption after birth,[31–39] micropuncture studies in rat and rabbit show that the neonatal late distal tubule has comparable[40] or approaching comparable[41] sodium reabsorption to the adult. However, these differences in maturation may also be channel-dependent. Whereas net sodium absorption is substantially increased in the late distal nephron, there is absence of net potassium secretion early in life with a temporal lag between sodium reabsorption and potassium secretion.[41] Based on our data showing an increased distal tubular cross-sectional staining relative to proximal tubular staining in the experimental preterm samples, we propose that the distal tubule may compensate for the proximal tubular delay in the preterm infant. However, it remains unknown if ongoing nephrogenesis is impacted by early compensatory hypertrophy or hyperplasia. Further studies are needed to delineate these mechanistic changes.

To date, the study of postnatal nephrogenesis in preterm humans relies on autopsy data and small sample sizes. These studies, including this one, cannot control the multitude of factors that these infants are exposed to during the neonatal intensive care unit stay and these uncontrolled variables may confound the data. Due to limited information in the autopsy report and without original medical records, we cannot comment on differences in nephrotoxic exposures or episodes of AKI that caused their premature cessation while allowing the others to continue. A lack of documentation does not equate to a lack of exposure. There were no documented cases of AKI in the autopsy report; however, AKI is often underrecognized in the neonatal intensive care unit population.[42] One notable theme in the autopsy report was the finding of BPD. Previous work in the rat has suggested impaired nephrogenesis secondary to hyperoxia, but no difference was seen in the nephron number at day 10.[43] Further work is needed to characterize the role of BPD, and its associated therapies, on nephrogenesis.

By narrowing our investigation to a PMA of 30 to 36 weeks, we learned that some infants continue nephrogenesis past 40 days. Therefore, our study provides new hope that many infants still do continue forming nephrons past this CA. Our data suggest a window of both vulnerability and potential protection beyond the first 40 days of life. Vigilance for kidney protective measures in the neonatal period cannot be overstated. Our evidence of ongoing nephrogenesis during the postnatal period opens the novel possibility for normal or near-normal nephron endowment in preterm infants if we can reduce the physiologic stress and negative exposures in the neonatal period.

DISCLOSURE

MPS has research funding through Otsuka which is not relevant to this work. JC, SY, LD, and KV have declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Figure S1. Perinatal definitions.

Figure S2. Example of region of interest for quantification.

Figure S3. Nephrogenesis cessation timing and cause of death.

Figure S4. Example of medullary ray count/glomerular generation.

Figure S5. Immunofluorescence of SIX1, SIX2, and WT1 for the 7 preterm samples.

Figure S6. Preterm infants with early PMA who survive greater than 40 days and younger PMA demonstrate greater compensation.

Figure S7. SLC12A3+ cross-sectional staining significantly increases with increasing postmenstrual age in preterm infants.

Figure S8. Assessment of SLC12A3 nuclear counts and area/nuclear counts of SLC12A3+ tubules support distal tubular hypertrophy.

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