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Podocyte Number in Children and Adults: Associations with Glomerular Size and Numbers of Other Glomerular Resident Cells

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ABSTRACT

Increases in glomerular size occur with normal body growth and in many pathologic conditions. In this study, we determined associations between glomerular size and numbers of glomerular resident cells, with a particular focus on podocytes. Kidneys from 16 male Caucasian-Americans without overt renal disease, including 4 children (≤3 years old) to define baseline values of early life and 12 adults (≥18 years old), were collected at autopsy in Jackson, Mississippi. We used a combination of immunohistochemistry, confocal microscopy, and design-based stereology to estimate individual glomerular volume (IGV) and numbers of podocytes, nonepithelial cells (NECs; tuft cells other than podocytes), and parietal epithelial cells (PECs). Podocyte density was calculated. Data are reported as medians and interquartile ranges (IQRs). Glomeruli from children were small and contained 452 podocytes (IQR=335–502), 389 NECs (IQR=265–498), and 146 PECs (IQR=111–206). Adult glomeruli contained significantly more cells than glomeruli from children, including 558 podocytes (IQR=431–746; $P<0.01$), 1383 NECs (IQR=998–2042; $P<0.001$), and 367 PECs (IQR=309–673; $P<0.001$). However, large adult glomeruli showed markedly lower podocyte density (183 podocytes per 10^6 μm^3) than small glomeruli from adults and children (932 podocytes per 10^6 μm^3; $P<0.001$). In conclusion, large adult glomeruli contained more podocytes than small glomeruli from children and adults, raising questions about the origin of these podocytes. The increased number of podocytes in large glomeruli does not match the increase in glomerular size observed in adults, resulting in relative podocyte depletion. This may render hypertrophic glomeruli susceptible to pathology.

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CKD is a global pandemic.[1] There are multiple causes of CKD, and although each cause of CKD shows a particular pathophysiology, most share common features. Arguably, the most significant of these is podocyte injury or dysfunction.[2]

Podocytes have a highly specialized structure and a very limited capacity to replicate under normal circumstances.[3] As major components of the glomerular filtration barrier, they fulfill a number of important functions, including structural support to capillary loops, synthesis of components of the glomerular basement membrane, synthesis and secretion of several cytokines and growth factors, and immunologic functions.[4] Consequently, podocyte injury comes at a high price for the glomerulus.

In recent years, the podocyte depletion hypothesis has emerged as a unifying concept in the pathogenesis

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of glomerular disease.[5] On the basis of multiple observations in animal models,[4,6–10] podocyte depletion can be defined as absolute when it involves a reduction in the total number of podocytes per glomerulus.[11,12] In a landmark study, Wharram *et al.*[5] showed that a 40% reduction in podocyte number is a direct cause of FSGS and therefor, long-standing CKD in rats. To date, a similar threshold has not been defined in humans.

Relative podocyte depletion occurs when the number of podocytes does not keep pace or match an increase in glomerular volume or filtration surface area. Glomerular size increases with normal body growth as well before and during multiple renal pathologies, including both diabetic nephropathy[13–17] and FSGS.[18–24] It has been suggested that an increase in glomerular size (also known as glomerular hypertrophy) is usually a compensatory mechanism that serves to match physiologic demands and sustain renal function.[25] Over the last decade, our group has described multiple factors associated with compensatory glomerular hypertrophy in humans without overt renal disease, including older age, low nephron number (N_{glom}), obesity, hypertension, and African-American race.[26] Interestingly, the boundary between compensatory and pathologic hypertrophy remains unclear and may be associated with relative podocyte depletion.

Despite the current interest in podocyte depletion, few studies to date have reported total numbers of podocytes in normal glomeruli.[27–32] This is, in part, because of the difficulty in counting podocytes and the lack of consensus on how to accurately count them. Lemley *et al.*[11] recently compared five methods for estimating podocyte number and recommended that the design–based dissector/optical fractionator method was the optimum technique when sufficient kidney tissue was available. This method provides an estimate of the volume of individual glomeruli as well as the total numbers of each of the resident glomerular cells, including podocytes, endothelial cells, mesangial cells, and parietal epithelial cells (PECs).[12] It, thus, provides an ideal tool to analyze both absolute and relative podocyte depletion and associations with numbers of other glomerular resident cell types.

In this study, we used design-based stereology to estimate glomerular volume and the numbers of podocytes and other resident glomerular cells in kidneys collected at autopsy in Caucasian-American boys and men. Our findings provide new insights into possible changes in glomerular cell populations in the context of glomerular hypertrophy in the nondiseased kidney, and they suggest that large adult glomeruli have relative podocyte depletion and therefore, may be susceptible to the development of pathology.

RESULTS

General Demographics

General demographics of four children are provided in Table 1. Briefly, all four children were 3 years of age or younger and presented no complications during pregnancy. Body surface area at the time of death ranged from 0.31 to 0.71 m^2. Causes of death for all children were not cardiovascular-related, and there were no signs of hypertension in these subjects. All children had adequate birth weights for gestational age and therefore, nephron number values>0.6 million,[33] both of which can serve as good surrogate markers of a normal fetomaternal environment.[26] These four subjects provided a baseline to identify changes in glomerular size and cellularity in adulthood.

General demographics for 12 adult subjects are provided in Table 2. The youngest adult was 25 years old, whereas the oldest adult was 49 years of age; 50% of the adult subjects were either overweight or obese (body mass index≥25.0 but <40 kg/m^2). Body surface area ranged from 1.45 to 2.64 m^2, N_{glom} ranged from 0.55 to 1.66 million, and N_{glom}/body surface area ranged from 1.06 to 0.55 million/m^2. Birth weight was normal (>2.5 and <4.0 kg[34]) in those subjects with available data (n=8). Five adults were hypertensive, and five adults had cardiovascular-related deaths. Additional information regarding pathologic analysis of these kidneys, including glomerulosclerosis, cortical fibrosis, and arteriosclerosis, is provided in Supplemental Table 1.

Glomerular Volumes and Cell Numbers

Individual glomerular volume (IGV) was estimated in a total of 480 glomeruli (30 per subject). The three smallest and the three largest glomeruli per subject (10th and 90th percentiles; n=96 glomeruli) were selected for additional cellular analysis. Podocytes were identified according to several criteria (Figure 1, A and A′), namely specific cytoplasmic immunostaining for Wilms' Tumor 1 (WT-1) (Figure 1, B and B′), presence of major cytoplasmic projections (Figure 1B′), and nuclear location outside capillary loops. When possible, lack of immunostaining for vWF (Figure 1, C and C′) was also used as part of these criteria. Because of the unexpected expression of WT-1 in the cytoplasmic compartment, we provide additional details regarding the specificity of this marker. Although WT-1 and Synaptopodin (SNP) were expressed within the same cells (Supplemental Figure 1), WT-1 was present in the cell body and major processes, whereas SNP was mostly located in foot processes. Furthermore, WT-1 was consistently found in the podocyte cytoplasm and tissue from nephrectomies and biopsies (Supplemental Figure 2).

Initially, we intended to use immunostaining for vWF to identify endothelial cells. However, vWF staining was not consistent in all autopsy samples, and we, therefore, classified all cells on the glomerular tuft that were not podocytes as nonepithelial cells (NECs; the majority of these cells were endothelial and mesangial cells). PECs were identified by their location on Bowman's capsule.

Glomerular Size Differs Significantly between Children and Adults

Representative light microscopic images of glomeruli from children and adults are provided in Figure 2, A and B, respectively. IGV estimates for 96 glomeruli in 16 subjects ranked by median IGV are shown in Figure 2C. Considerable variation in glomerular size was present in children, but this was far greater

Table 1. Demographic data for four male Caucasian-American children without kidney disease

Rank[a]	Age (yr)	GA (wk)	BS Area (m^2)	N_{glom} (million)	N_{glom}/BS Area (million/m^2)	Birth Weight (kg)	Hypertensive Status	COD	CVRD
Ch1	0.25	35	0.36	1.11	3.08	2.92	Normotensive	SIDS	No
Ch2	0.50	39	0.31	0.84	2.71	3.54	Normotensive	Other	No
Ch3	3.00	38	0.71	1.22	1.72	2.80	Normotensive	Accident	No
Ch4	3.00	41	0.68	0.90	1.32	3.63	Normotensive	Accident	No
Median	1.75	38.50	0.52	1.01	2.22	3.23	0%	NA	0%
IQR	0.3–3.0	35.8–40.5	0.3–0.7	0.9–1.2	1.4–2.9	2.8–3.6	NA	NA	NA

GA, gestational age at birth; BS area, body surface area; N_{glom}, total nephron number; COD, cause of death; CVRD, cardiovascular-related death; Ch, child; SIDS, sudden infant death syndrome; Other, hematologic, neoplastic, or infectious; NA, not available.
[a]Subjects were ranked on the basis of median IGV.

Table 2. Demographic data for 12 adult Caucasian-American men without kidney disease

Rank[a]	Age (yr)	BMI (kg/m^2)	BS Area (m^2)	N_{glom} (million)	N_{glom}/BS Area (million/m^2)	Birth Weight (kg)	Hypertensive Status	COD	CVRD
A1	43	18.19	1.57	1.66	1.06	3.38	Hypertensive	Other	No
A2	25	18.10	1.45	0.69	0.48	NA	Normotensive	Other	No
A3	31	30.80	1.86	1.05	0.57	3.25	Normotensive	Accident	No
A4	41	34.02	2.36	1.12	0.47	3.86	Normotensive	CAD	Yes
A5	41	23.02	1.80	0.83	0.46	3.66	Normotensive	Other	No
A6	25	23.80	1.72	0.95	0.55	NA	Normotensive	Cardiac, not CAD	Yes
A7	47	22.75	1.81	1.15	0.64	NA	Normotensive	Accident	No
A8	48	20.54	1.94	1.00	0.52	NA	Normotensive	Other	No
A9	37	36.36	2.17	0.93	0.43	3.29	Hypertensive	CAD	Yes
A10	49	35.88	2.31	0.78	0.34	3.12	Hypertensive	CAD	Yes
A11	37	39.42	2.64	0.76	0.29	3.23	Hypertensive	Other	No
A12	39	26.70	2.01	0.55	0.27	3.46	Hypertensive	CAD	Yes
Median	40	25.53	1.9	0.94	0.47	3.34	42%	NA	42%
IQR	33–46	22.8–35.4	1.7–2.3	0.8–1.1	0.4–0.6	3.2–3.6	NA	NA	NA

BMI, body mass index; BS area, body surface area; N_{glom}, total nephron number; COD, cause of death; CVRD, cardiovascular-related death; A, adult; Other, infectious, hematologic, neoplastic, or central nervous system-related but not cardiovascular or pulmonary; NA, not available; CAD, coronary artery disease.
[a]Subjects were ranked on the basis of median IGV.

in adults. Adults showed greater aggregated median IGV (Figure 2D) ($P<0.001$) and IGV variance (Figure 2E) ($P<0.01$) than children. Median IGV in children was 0.41×10^6 μm^3 (interquartile range [IQR]=0.29×10^6 μm^3–0.64×10^6 μm^3), and in adults, it was 2.42×10^6 μm^3 (IQR=1.54×10^6 μm^3–3.27×10^6 μm^3), a 5.9-fold difference. Figure 2F shows the marked right shift in the adult IGV distribution compared with that in children. The smallest glomerulus observed in children had a volume of 0.21×10^6 μm^3, whereas the largest had a volume of 0.89×10^6 μm^3—a difference of 0.68×10^6 μm^3 or 4.2-fold. In contrast, the smallest glomerulus in adults had a volume of 0.76×10^6 μm^3, whereas the largest had a volume of 6.91×10^6 μm^3, a difference of 6.15×10^6 μm^3 or 9.1-fold.

Numbers of Glomerular Cells Differ Significantly between Children and Adults

Representative confocal images showing the marked differences in glomerular cellularity between children and adults are shown in Figure 3, A and B. Aggregated data for total numbers of NECs, podocytes, and PECs in children (n=24 glomeruli) and adults (n=72 glomeruli) are reported in Figure 3, C and D, and they clearly show the greater variability in adult cell numbers. In adult glomeruli, the median podocyte count was 558 (IQR=431–746; 3.7-fold), whereas median NEC and PEC counts were 1383 (IQR=998–2042; 7.0-fold) and 367 (IQR=309–673; 10.3-fold), respectively. In children, the median podocyte count was 452 (IQR=335–502; 2.1-fold), whereas median NEC and PEC counts were 389 (IQR=265–498; 4.2-fold) and 146 (IQR=111–206; 2.8-fold), respectively.

Numbers of Glomerular Cells in the Context of Glomerular Hypertrophy

The estimation of absolute numbers of specific cell types in glomeruli of known volume allowed us to examine relationships between glomerular size and numbers of each cell type. As seen in Figure 4A, IGV was directly and strongly associated with the numbers of NECs (aggregated R=0.96, children R=0.83, adult R=0.92; $P<0.001$ in each case). In a linear regression analysis of aggregated glomeruli (from children and adults), 92% of IGV variability was explained by numbers of NECs (F=1084; $P<0.001$), predicting an increase of 544 NECs per 10^6 μm^3 glomerular tuft volume. Similarly, IGV was

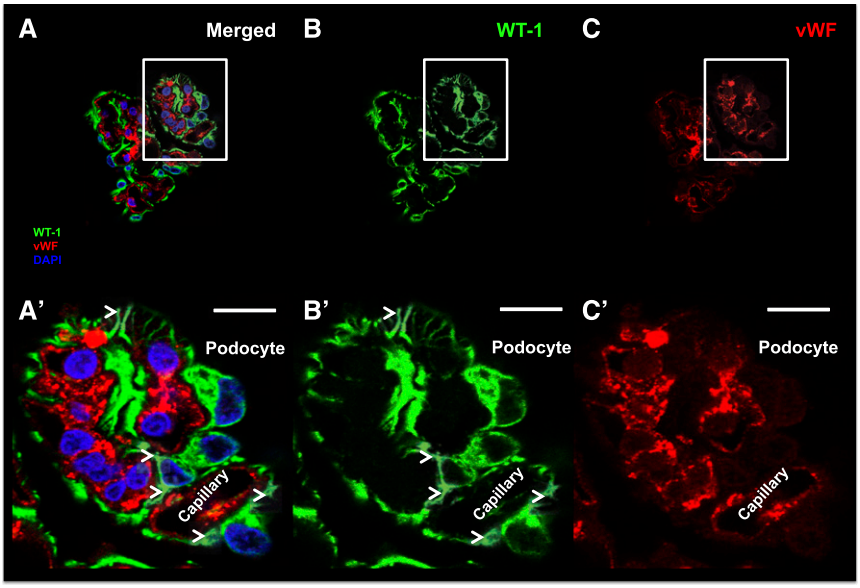


Figure 1. Podocyte identification. (A) A representative confocal image of a glomerular tuft is shown (merged) using (B) WT-1 (green; specific podocyte marker), (C) vWF (red; specific endothelial cell marker), and DAPI (blue; nuclear marker). The corresponding insets show our podocyte identification criteria: (A′) expression of WT-1 in podocyte cytoplasm, (B′) lack of expression of vWF, and (C′) their location outside capillaries. Arrowheads show classic podocyte morphology (major projections). Scale bars, 10 μm. DAPI, 4′,6-diamidino-2-phenylindole.

closely directly associated with PEC number (aggregated R=0.88, children R=0.78, adults R=0.86; P<0.001 in each case) (Figure 4B). A linear regression model predicted an increase of 141 PECs per 10^6 μm^3 glomerular tuft volume (R^2=0.79; F=357; P<0.001).

Unlike the situation with PECs and NECs, the association between IGV and podocyte number (Figure 5A) was weaker in adults than in children (aggregated R=0.70, children R=0.87, adults R=0.76; P<0.001 in each case). The adult relationship between number of podocytes and IGV was better fitted in a quadratic model (Figure 5A), with podocyte number plateauing with IGV values approximately $>4\times10^6$ μm^3. Because of the great variability in IGV in adults, we also examined the relationships between podocyte number and glomerular size in children and between tertiles of glomerular size in adults. As shown by Figure 5B, glomeruli from children contained the same number of podocytes as small and medium adult glomeruli (P>0.05). However, large adult glomeruli (tertile 3) contained significantly more podocytes than glomeruli from children and small and medium adult glomeruli (P<0.01). Figure 5C shows that adult glomeruli (all three tertiles) had lower podocyte densities (podocyte number per 10^6 μm^3 glomerular tuft volume) than glomeruli from children (P<0.01 for small glomeruli; P<0.001 for medium and large glomeruli). Large adult glomeruli had a significantly lower podocyte density than small and medium adult glomeruli (P<0.05 for medium glomeruli; P<0.001 for small glomeruli).

Cell Ratios in the Context of Glomerular Hypertrophy

Figure 6A illustrates differences in the trajectories of numbers of NECs and podocytes in the context of IGV (F=550; P<0.001). Although small glomeruli had similar numbers of NECs and podocytes (indeed, podocytes slightly outnumbered NECs), in adult glomeruli, NECs outnumbered podocytes (Figure 6B), and this was most pronounced in large adult glomeruli, in which the NEC-to-podocyte ratio was close to 3:1. In contrast, the trajectories of both PEC number and podocyte number with IGV were not statistically different (P>0.05) (Figure 6C). However, the ratio of PECs/podocyte was significantly higher in adult glomeruli than children glomeruli (1:3; P<0.001 for all tertiles) and large (1:1) compared with small (1:2) adult glomeruli (Figure 6D).

Histologic Evidence of Possible Sources of Podocytes

WT-1+PECs (also known as parietal podocytes) were found in approximately 90% of glomeruli in both children and adults. These cells were typically found close to the vascular pole and never observed near the tubular pole (Figure 7, A and A′). WT-1+cells were also occasionally observed in the tunica intima and the tunica media of arterioles in the juxtaglomerular apparatus (JGA) (Figures 7, B and B′).

Zonal Analyses in Adult Glomeruli

From 72 sampled adult glomeruli, 31 glomeruli were located in the outer cortex (superficial), 24 glomeruli were located in the middle cortex, and 17 glomeruli were located in the inner cortex (juxtamedullary). There were no zonal differences in IGV, podocyte number, or podocyte density (Supplemental Figure 3).

DISCUSSION

The three major findings of this study were (*1*) glomerular size and numbers of glomerular cells vary widely in children and adults without overt kidney disease, with greater variation seen in adults; (*2*) large adult glomeruli contain more podocytes than glomeruli from four young children and small adult glomeruli; and (*3*) these large adult glomeruli have a lower podocyte density, which may leave them in a setting of relative podocyte depletion.

Glomerular hypertrophy plays pivotal roles in the development of FSGS[2,18,19] and the progression of diabetic nephropathy,[35–38] two of the most common causes of CKD. According to the hyperfiltration theory,[25] an imbalance between glomerular mass and physiologic requirements drives glomerular

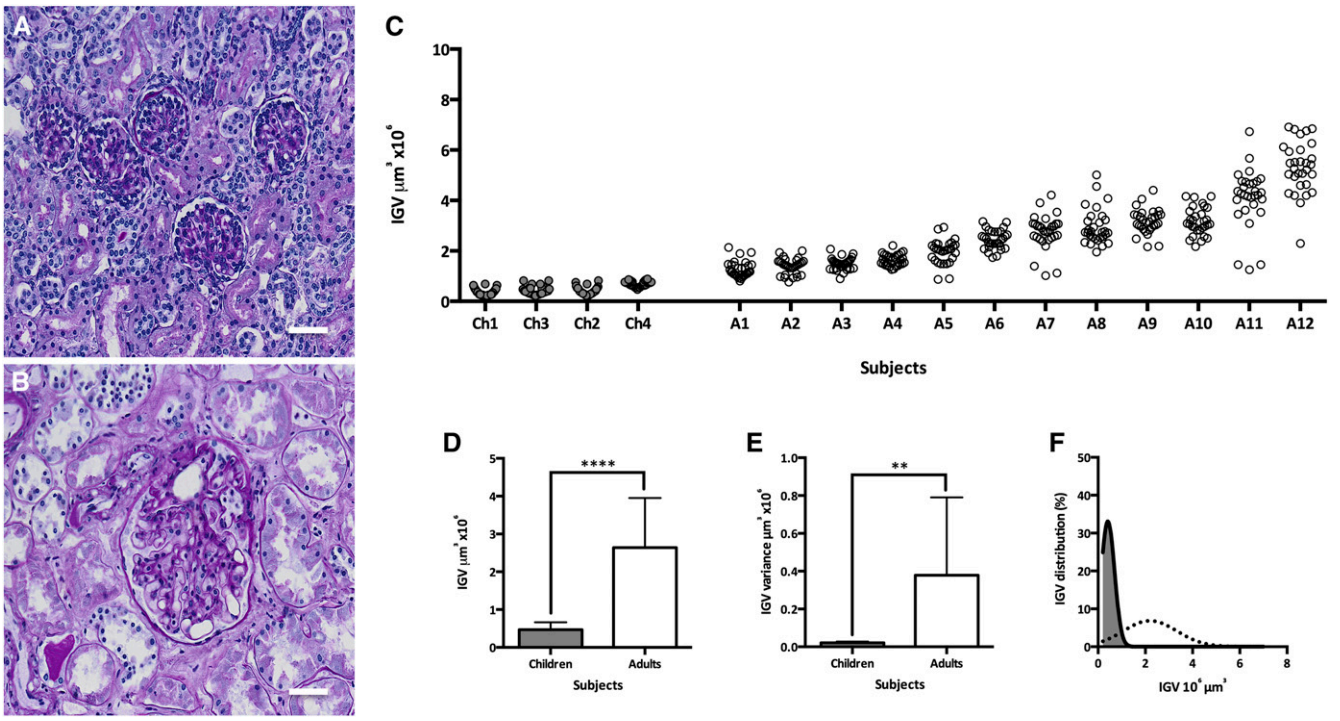


Figure 2. Glomeruli from adults are larger and have more variable size than glomeruli from children. A and B show representative images of average glomeruli from children and adults, respectively, using periodic acid–Schiff staining. (C) IGV values in 16 Caucasian-American men and boys. Each circle represents one glomerulus, and each column represents one subject with 30 glomeruli per subject; Ch1–Ch4 represent four young children (gray), and A1–A12 represent 12 adults (white) ranked by median IGV. (D) Aggregated IGV differences between children and adults. (E) Difference in IGV variance per subject between glomeruli from children and adults. (F) IGV distributions in children (solid line with gray area) and adults (dotted line). Bars represent median values with IQR. Scale bars, 50 μm. $^{**}P<0.01$; $^{****}P<0.001$.

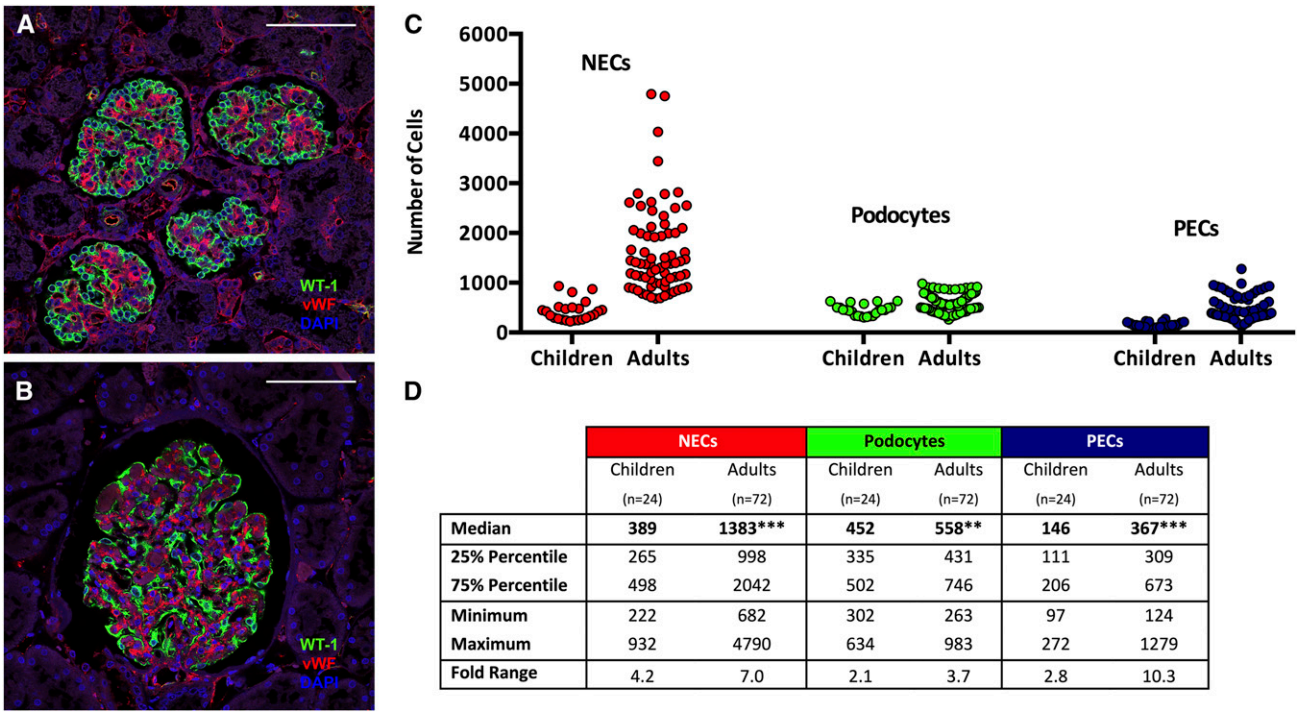
hypertrophy as an appropriate compensatory response to sustain renal function. We hypothesize that human glomerular growth is initially a healthy compensatory step, which if exaggerated, can result in pathology.

Human glomeruli have been reported to increase in size up to 7-fold from infancy to adulthood.[39,40] These findings confirm this significant increase in glomerular size from childhood to adulthood, although it is important to note that kidneys from only four children were analyzed in this study. We report a 5.9-fold increase in glomerular volume between childhood and adulthood and show a much greater variability in glomerular sizes in adults than in children. This latter finding suggests that the great variability in IGV found within and between adults may be established later in life.[41] We have previously reported that increased IGV median and variance in adult American men are closely associated with low nephron number,[33] older age,[42] obesity,[43] African-American race,[44] and hypertension.[45] This study suggests that glomerular hypertrophy is initially associated with body growth from childhood to adulthood. Additional adult hypertrophy occurs in association with aging, nephron loss, hypertension, and obesity—all variables that can be present individually or combined, reflecting the multifactorial nature of glomerular hypertrophy.[41,46]

To date, only a handful of studies have estimated total podocyte number in adult human glomeruli, and these studies focused on pathology. Studies of type 2 diabetes identified absolute podocyte depletion in the early stages of diabetic nephropathy, which was closely related to disease progression.[27–30] Absolute podocyte depletion was also reported in studies of IgA nephropathy and hypertensive nephrosclerosis.[31,32] Although these studies provided valuable insights into the association between podocyte number and the development and progression of renal disease, our study shows, for the first time, that podocyte number varies within and between subjects without overt renal disease.

In this study, median podocyte number per adult glomerulus was 558, with the lowest count being 263 podocytes and the highest count being 983 podocytes (3.7-fold range). This finding raises two important questions. (*1*) When is podocyte number determined? (*2*) What is the optimal number of podocytes required in an adult glomerulus? These questions are considered in turn.

These findings provide several novel insights into the establishment of adult podocyte number. Glomeruli in four young children contained fewer podocytes than large adult glomeruli; >30 years ago, Olivetti *et al.*[47] described a similar pattern between young, adult, and uninephrectomized rats. Taken together, these findings suggest that additional podocytes may be acquired by certain glomeruli after early childhood. The



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	NECs		Podocytes		PECs	
	Children (n=24)	Adults (n=72)	Children (n=24)	Adults (n=72)	Children (n=24)	Adults (n=72)
Median	**389**	**1383*** **	**452**	**558****	**146**	**367*** **
25% Percentile	265	998	335	431	111	309
75% Percentile	498	2042	502	746	206	673
Minimum	222	682	302	263	97	124
Maximum	932	4790	634	983	272	1279
Fold Range	4.2	7.0	2.1	3.7	2.8	10.3

Figure 3. Large glomeruli from adults contained more podocytes, NECs and PECs than glomeruli from children. A and B show representative confocal images of glomeruli from children and adults, respectively, with WT-1 (green), vWF (red), and DAPI (blue). C and D show the numbers of NECs, PECs, and podocytes per glomerulus from children and adults. C illustrates the variability of each cell type between glomeruli from adults and children; each circle represents the value for a single glomerulus,. D provides details of the medians, IQRs (25% and 75% percentiles), minimum and maximum values, and fold ranges per group. Scale bars, 100 μm. DAPI, 4′,6-diamidino-2-phenylindole. $^{**}P<0.01$; $^{***}P<0.001$.

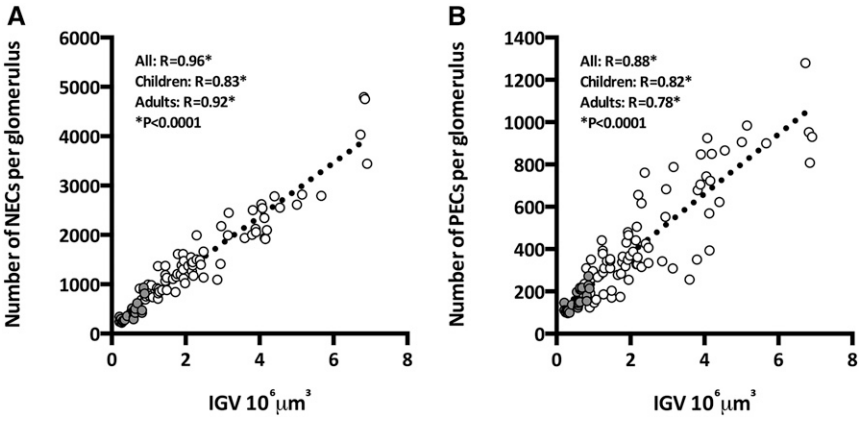

Figure 4. Glomerular size is closely associated with numbers of NECs and PECs. (A) Association between IGV and numbers of NECs in glomeruli from children (gray) and adults (white). (B) Association between IGV and PECs in glomeruli from children (gray) and adults (white).

origin of these additional podocytes remains unknown, but in recent years, there has been great interest in the topic of podocyte replacement in the postnatal period.

Mature podocytes exit the cell cycle to take on a terminally differentiated phenotype and express cyclin–dependent kinase inhibitors, such as p16INK4a, P21Cip1, P27Kip1, and P57Kip2.[48–52] After this point, podocytes are considered incapable of undergoing mitosis under normal conditions, although podocyte proliferation occurs in certain pathologic conditions, such as HIV-associated nephropathy.[53,54] Podocytes can be induced to proliferate *in vitro* when cultured from freshly isolated glomeruli,[55] but these cells express low levels of many of the podocyte–specific differentiation markers, suggesting that podocytes are only capable of proliferating after they have dedifferentiated to a certain degree. Recent evidence suggests that progenitor cells residing in the JGA[56] and Bowman's capsule (PECs)[57–61] may give rise to podocytes. Other lines of evidence suggest that new podocytes may arise from bone marrow.[62–64] In this study, WT-1+PECs (parietal podocytes) were observed near the vascular pole in most glomeruli in children and adults, and some WT-1+cells were occasionally observed in the tunica intima and the tunica media of arterioles in the JGA. Whether any of these cells give rise to new podocytes deserves additional attention.

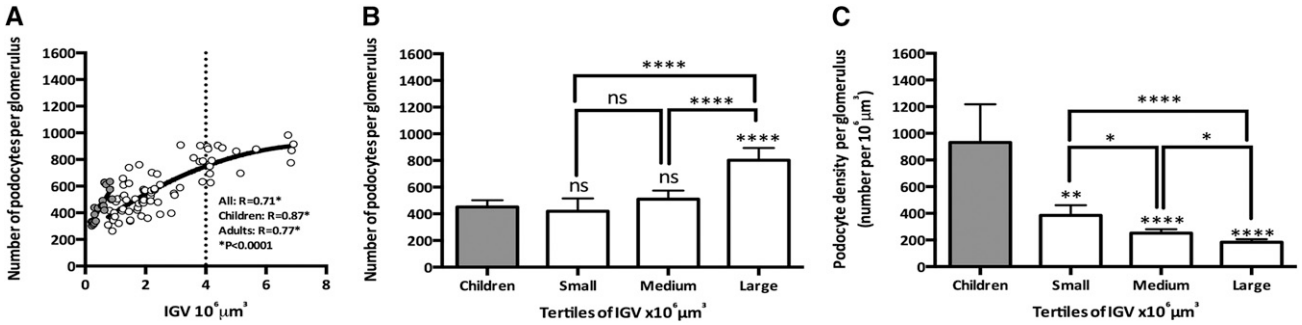

Figure 5. Large adult glomeruli have more podocytes and lower podocyte density than smaller adult glomeruli and glomeruli from children. (A) IGV and numbers of podocytes in children (gray circles) and adults (white circles). The solid line represents the line of best fit in children (linear) and adults (quadratic), and the dotted line shows the beginning of the curve plateau at 4×10^6 μm^3. (B) Number of podocytes in children and adult IGV tertiles (small, medium, and large glomeruli). (C) Podocyte density in children and adult IGV tertiles (small, medium, and large glomeruli). Children: aggregated data from four infants (6 glomeruli per subject; n=24 glomeruli); adult IGV tertiles were tertile 1 (small glomeruli), between 0.76 and 1.72×10^6 μm^3; tertile 2 (medium glomeruli), between 1.74 and 2.86×10^6 μm^3; and tertile 3 (large glomeruli), between 2.94 and 6.91×10^6 μm^3. P values directly over the bars represent comparisons between children (gray bars) and each adult tertile. Bars represent median values with IQR. *P<0.05; **P<0.01; ****P<0.001.

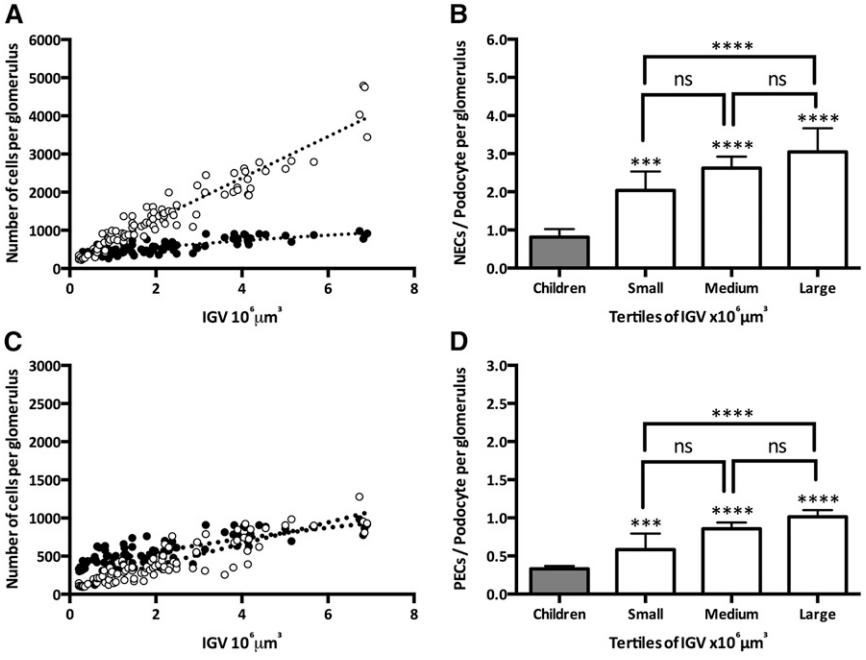

Figure 6. Numbers of NECs, PECs, and podocytes in the context of glomerular volume. (A) Numbers of podocytes (black circles) and NECs (white circles) in the context of IGV. (B) NEC-to-podocyte ratio in glomeruli from children and adults by adult IGV tertiles. (C) Numbers of PECs (black circles) and podocytes (white circles) in the context of IGV. (D) PEC-to-podocyte ratio in glomeruli from children and adults by adult IGV tertiles. P values directly over the bars represent comparisons between children (gray bars) and each adult tertile. Bars represent median values with IQR. ***P<0.001; ****P<0.001.

There is much interest in whether PECs can give rise to podocytes.[59,65] Wanner *et al.*[66] recently showed that podocyte generation is mainly active during glomerular development and may occur after acute glomerular injury but was not observed in aging kidneys or in response to nephron loss. Interestingly, a recent study by Berger *et al.*[67] showed that parietal podocytes (PECs expressing podocyte markers) disappeared from Bowman's capsule as glomeruli gradually underwent physiologic hypertrophy, suggesting that there is a functional podocyte reserve that directly differentiates into podocytes on Bowman's capsule. Berger *et al.*[68] proposed that this is explained by the lack of space to accommodate podocytes on the small glomerular tuft of a young child. This hypothesis is supported by our findings that show that glomeruli from young children are small and replete with podocytes (high podocyte numerical density) but have a lower podocyte number than large glomeruli from adults.

A novel method on the basis of flow cytometry was used recently to report a 7% increase in podocyte number after acute podocyte injury.[66] This is an interesting approach that has many advantages (*i.e.*, does not rely on time-consuming techniques). However, we believe that stereologic methods may also provide an important perspective. For example, stereology-based approaches consider the context of glomerular size and cortical location, both of which have significant value. We believe that this highlights how histologic/stereologic approaches, even if laborious and time consuming, are still valuable tools for the study of podocyte biology. Additional studies, especially combining lineage-tracing models and unbiased stereology, are urgently needed to quantify the efficiency of glomerular podocyte gain and possible modifiable mechanisms.

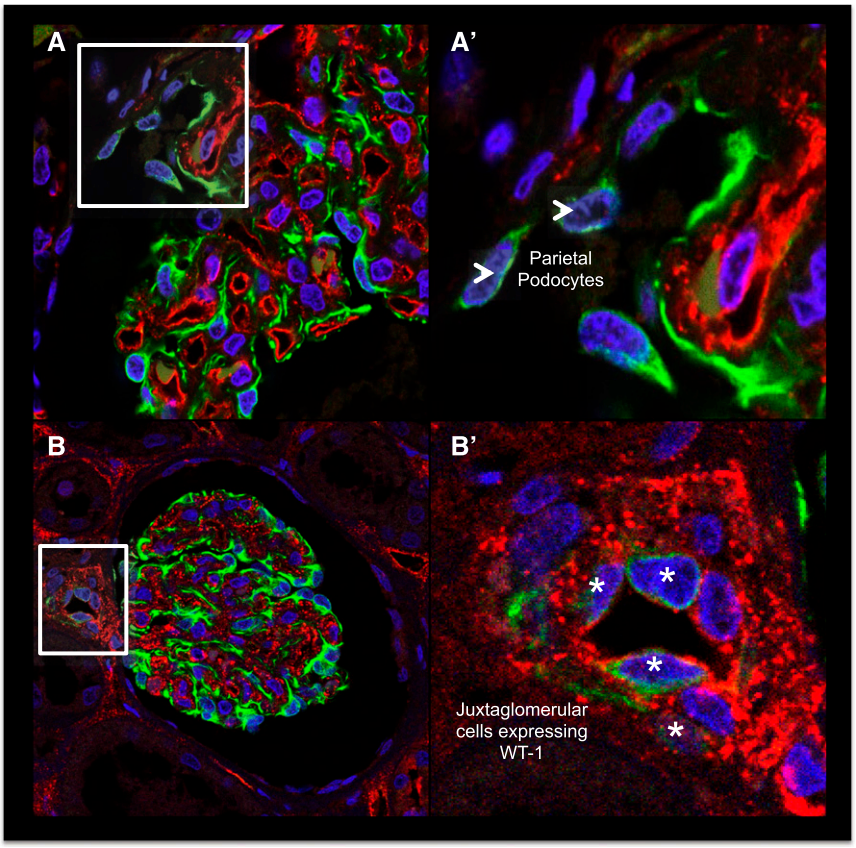

Figure 7. Possible podocyte sources in the human kidney. (A and A′) Parietal podocytes: PECs expressing WT-1 are located close to the vascular pole of a glomerulus (arrowheads). (B and B′) WT-1+cells were also occasionally observed in the tunica intima and the tunica media of arterioles in the JGA (asterisks).

To our knowledge, we present for the first time the concept of human podocyte endowment, a term that refers to the number of podocytes with which we are born. Although the sample size of children in this study is small, we believe that this concept is important and follows a series of contributions by our group to understand the role of nephron endowment in the risk, development, and progression of renal disease.[26,33,69–75] On the basis of the variation in podocyte number in children and adults, we postulate that some glomeruli at birth may be better equipped to deal with glomerular stress than others. It is evident that a more comprehensive analysis of the numbers of podocytes in newborns and young children is warranted, especially to find associations with low birth weight, prematurity, and adverse fetomaternal environments. We have commenced studies to answer this critical question.

With the recent interest in the role of podocyte depletion in glomerular pathology, the question arises as to the optimum number of podocytes required in a glomerulus to maintain glomerular health. Wharram *et al.*[5] defined 40% podocyte depletion as the threshold for established glomerular pathology in a rat model of podocyte injury. Earlier, Lemley *et al.*[31] reported that a similar degree of podocyte loss (approximately one third) marked the deterioration of glomerular structure and function in patients with IgA nephropathy. However, there is still no consensus on a reliable cutoff value for absolute podocyte depletion in the human kidney. Although this study has revealed a 3.7-fold variation in podocyte number per adult glomerulus, at the moment, we cannot provide a set value for an optimal podocyte number.

Despite containing more podocytes, hypertrophic adult glomeruli showed a substantial decrease in podocyte density, which may already mark an increased risk of glomerulosclerosis. The aim of glomerular hypertrophy is to increase filtration surface area to sustain renal function.[76] Previous studies in animal models and human tissue have shown that glomerular hypertrophy is closely associated with endothelial or mesangial cell hyperplasia.[77–79] Therefore, it is not surprising to find a strong association between glomerular volume and numbers of NECs, which included endothelial[80] and mesangial[81] cells. A recent study by Fukuda *et al.*[82] showed the critical importance of podocyte hypertrophy when compensating during glomerular hypertrophy. This study provided evidence that a possible pathway for pathologic glomerular hypertrophy was a mismatch between the volume of podocytes and glomerular volume.[82] Our study also indicates that, in hypertrophic human glomeruli, there is an increase in the NEC-to-podocyte ratio, providing another piece of evidence to support clear differences in replication potential of each cell population in the context of glomerular hypertrophy. Taken together, we propose that there is evidence of relative podocyte depletion in hypertrophic glomeruli and that additional studies in pathologic tissue will provide critical evidence to define a threshold of podocyte density marking the development of glomerulosclerosis.

Interestingly, PECs have gained much attention in recent years because of their role in the development of glomerulosclerosis.[57,59,83,84] Activated PECs can avidly proliferate, migrate, and produce extracellular matrix.[85] To our knowledge, this study provides the first estimates of total PEC numbers in human glomeruli. The numbers of PECs were highly variable in adult glomeruli (up to a 10.3-fold range) and closely associated with glomerular size. The biologic significance of higher numbers of PECs in hypertrophic glomeruli remains unclear, but multiple hypotheses can be proposed, including (*1*) preservation of a physical barrier during glomerular growth, (*2*) limited podocyte gain to cope with increases in the filtration surface area, and (*3*) early PEC activation.

Although subjects included in this study come from the largest and most comprehensive kidney autopsy series in the world, this study has several limitations. We acknowledge the inherent limitation of a cross-sectional study design. We emphasize that, although the number of young children included in this study is small, we used all available subjects that met our inclusion criteria: men, Caucasian Americans, younger than 3 years of age, adequate birth weight for gestational age, and no intercurrences during pregnancy. Another limitation was the inability to present data for endothelial and mesangial cells separately because of the suboptimal vWF immunostaining in some of the autopsy samples. We would advise caution before extrapolating these findings to different cohorts (*i.e.*, women and African Americans). Finally, our selection of glomeruli for cell counting allowed us to compare small and large glomeruli. This sampling strategy assumes that the relationship between glomerular size and cell numbers is continuous across the range of glomerular volumes.

In conclusion, this study provides evidence of (*1*) glomerular hypertrophy in adult glomeruli and a strong direct association between glomerular size and numbers of NECs; (*2*) higher numbers of podocytes in large adult glomeruli that may reflect both podocyte endowment at birth and podocyte gain in the postnatal period; and (*3*) relative podocyte depletion in large adult glomeruli that may increase their risk to develop glomerulosclerosis.

CONCISE METHODS

Subject Selection

Sixteen Caucasian-American men and boys were selected for study: four children (≤3 years old) and 12 adults (≥18 years old) without renal disease. The four children presented no complications during pregnancy and had adequate birth weights for gestational age. Adult subjects presented different CKD risk factors (older age, low N_{glom}, high body surface area, hypertension, or cardiovascular-related deaths). Kidneys were obtained from autopsies performed at the University of Mississippi Medical Center (Jackson, Mississippi). Ethics approval was obtained in advance from the Institutional Review Board of the University of Mississippi Medical Center and the Monash University Human Research Ethics Committee. On collection, kidneys were perfusion fixed with 10% buffered formalin, bisected, and then immersed in 10% formalin for 10 days. Representative kidney blocks from the upper pole and midportion of the kidney were embedded in paraffin as previously described.[40]

Measurement of Pathologic Parameters

The methodology to assess pathology parameters has been described in previous publications.[73,86] Briefly, representative kidney blocks from the upper pole and the midportion of each kidney were paraffin embedded. Sections were cut at 4 μm and stained with periodic acid–Schiff-hematoxylin and picrosirius red stains for fibrillar collagen. Measurement of the percentage of glomerulosclerosis and arterial intimal thickening (arteriosclerosis) was performed on periodic acid–Schiff-hematoxylin–stained sections. The percentage of sclerotic glomeruli was estimated by counting sclerosed and nonsclerosed glomerular cross-sections in nonoverlapping ×100 microscopic fields moving from the superficial to the inner cortex, with at least 400 glomerular cross-sections being sampled per subject. The severity of arteriosclerosis was measured as a ratio of the thickness of the intima to the outer wall diameter at a magnification of ×400 in interlobular arteries 90–250 μm in diameter using the linear measurement function of Image-Pro Plus morphometric software (Media Cybernetics Inc., Bethesda, MD). With the same software, cortical fibrosis was measured in ×200 images as the proportion of cortex staining red with the picrosirius stain.

Estimation of Glomerular Volume and Cell Numbers

We estimated IGVs and the absolute numbers of podocytes, NECs, and PECs per glomerulus using a previously described design–based stereologic approach.[12] This method is considered the gold standard for quantification of podocyte number and glomerular volume when sufficient tissue is available.[11] In brief, 50 serial paraffin sections at 14-μm thickness were obtained for each kidney. Using these sections, 30 glomeruli (10 glomeruli each from the outer, middle, and inner cortexes) per subject were sampled using physical disectors.[41,42] Section profiles of these 30 sampled glomeruli were then imaged using an Olympus DotSlide system, generally providing between 8 and 16 profiles per glomerulus. Glomerular profiles were labeled with a flag and a unique identifier. These virtual images served as maps to find all profiles of each of these glomeruli during confocal microscopy. The volumes of all 30 sampled individual glomeruli per kidney were estimated using the Cavalieri estimator.[87]

Every second section (in adults) and every section (in children) were then immunostained to facilitate cell identification and counting. Sections were immunostained using an antibody against WT-1 antigen (monoclonal mouse anti-human WT-1; M356101, clone 6F-H2 [1:50]; for podocyte identification; DAKO) and an antibody against vWF (1:200), which in this case, was a polyclonal rabbit anti-human vWF (A008202; for endothelial cell identification; DAKO). In this study, WT-1 immunostaining was found exclusively in podocyte cytoplasm. Although immunostaining for WT-1 is most often reported to be nuclear, it is well known that WT-1 isoforms are present in the nuclei and cytoplasm of many cell types, including mouse mesonephros, mouse mesothelioma, and differentiated embryonic stem cells.[88] This localization of WT-1 immunostaining in podocyte cytoplasm confirms the findings of the work by Su *et al.*,[30] which used this same antibody and reported immunostaining of human podocyte cytoplasm, and agrees with the manufacturer's (DAKO) specifications regarding this antibody. To further validate this WT-1 antibody as a specific marker of podocyte cytoplasm, we performed double-labeled immunostaining for WT-1 and SNP, a well characterized podocyte marker. Both WT-1 and SNP were localized in the cytoplasm of the same cells (Supplemental Figure 1). To ensure that the WT-1 immunostaining of podocyte cytoplasm in our autopsy samples was not an artifact of postmortem autolysis, we tested nephrectomy and biopsy samples from our human tissue bank. In every case, specific immunostaining of podocyte cytoplasm was observed (Supplemental Figure 2).

Cells were counted in 96 glomeruli, which were comprised of 6 glomeruli from each of 16 subjects. The 6 glomeruli analyzed per subject were the 3 smallest and the 3 largest (representing the 10th and 90th percentiles, respectively) from 30 glomeruli per subject used for IGV estimation. Every immunostained section from each of six subsampled glomeruli per subject was imaged with a Leica SP5 laser confocal

microscope (Leica MicroSystems, Manheim, Germany). Optical dissectors were used to sample and therefore, count cells in 8 of 14 μm available for each glomerular profile (we did not count cells in a 3-μm guard region at the top and bottom of each section as detailed in ref. 12). Cells were sampled and counted using optical dissectors on the series of 1-μm confocal optical images, stacked as a virtual slide, and opened using an ImageJ[89] macro. After all newly appearing nuclei had been identified, we defined podocytes (tuft cells WT-1$^+$ and vWF$^-$), NECs (tuft cells WT-1$^-$), and PECs (cells located on Bowman's capsule). Podocyte density was calculated by dividing absolute podocyte number per glomerulus by IGV. Cell number ratios were calculated by dividing total numbers of NECs by total numbers of podocytes (NEC-to-podocyte ratio) and total numbers of PECs by total numbers of podocytes (PEC-to-podocyte ratio).

Subjects with the diagnosis of FSGS were excluded from any stereologic analysis in this autopsy cohort. Importantly, this method allowed the identification of glomeruli with signs of segmental sclerosis,[12] which were excluded from this study.

Statistical Analyses

Data were analyzed using GraphPad Prism, version 5.04 for Windows (La Jolla, CA) and StataCorp. (Statistical Software: Release 8; StataCorp., College Station, TX). Values are expressed as medians±IQRs unless otherwise stated. Mann–Whitney *U* test was used to compare variables with skewed distributions, and Spearman rank coefficient was used as a measurement of associations. *F* tests were applied to assess if lines of best fit were different. Linear regression analysis was performed using IGV as an independent variable and numbers of NECs, PECs, and podocytes as dependent variables. In all instances, a P value<0.05 was considered statistically significant.

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DISCLOSURES

None.

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