Human podocyte depletion in association with older age and hypertension

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Podocytes are postmitotic cells with a very limited capacity for regeneration. The extent of podocyte injury/loss may determine glomerular survival (29). The podocyte depletion paradigm has emerged in recent years as a unifying principle in the pathogenesis of glomerular pathology (66). Podocyte depletion can be defined as “absolute,” based on a reduction in the total number of podocytes per glomerulus, or “relative,” as occurs when the number of podocytes does not keep pace with increases in glomerular volume or filtration surface area (35, 49). Both absolute and relative podocyte depletion can directly lead to the development of glomerulosclerosis and progressive CKD (12, 15, 26, 27, 30, 39, 56).

Glomerular hypertrophy occurs before and during multiple renal pathologies, including diabetic nephropathy (10, 18, 45, 46, 64) and focal segmental glomerulosclerosis (FSGS) (8, 25, 34, 42, 43, 61, 68). It has been suggested that glomerular hypertrophy may be a compensatory mechanism that serves to meet altered physiological demands and maintain renal function (7). Over the last decade, our group has described multiple glomerular stressors in people without overt renal disease, which include older age and hypertension (23, 24, 51). However, whether these factors are associated with podocyte depletion remains unclear.

Despite the current interest in podocyte depletion, few studies have reported total numbers of podocytes in glomeruli from subjects without glomerular disease (11, 36, 38, 47, 60, 62). Studies of normal human kidneys are essential to understand pathophysiological changes that occur before the onset of disease. In the present study, we initially asked whether older age and hypertension were associated with podocyte depletion. However, in humans, this question is difficult to answer, mainly because of the close link between the two variables. To overcome this, we examined the independent and combined effects of older age and hypertension on human podocyte depletion. We used gold-standard design-based stereology (48, 49) to assess podocyte depletion in kidneys collected at autopsy from 19 male Caucasian American adults. Our results suggest that in adults without overt renal disease, older age is associated with absolute and relative podocyte depletion, whereas hypertension is only associated with relative podocyte depletion. Not surprisingly, podocyte depletion is more marked when both older age and hypertension are present.

MATERIALS AND METHODS

Subject selection. Kidneys from 19 adult male Caucasian Americans without overt renal disease were selected from our tissue bank. Tissue was obtained at autopsies performed at the University of Mississippi Medical Center in Jackson, MS. Ethics approval was
obtained in advance from the Institutional Review Board of the University of Mississippi Medical Center and Monash University Human Research Ethics Committee. Upon 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 (22), and used for histopathological analysis and quantification of glomerular cell numbers.

**Definition of hypertension.** Blood pressure measurements without medication were obtained for eight subjects. Measurements from terminal hospital admissions were not used unless patients were diagnosed as hypertensive and blood pressure was elevated. Mean arterial blood pressure was calculated from an average of at least three blood pressure measurements. Patients were categorized on the basis of a history of hypertension, consistently elevated blood pressure (≥140/90 mmHg), the presence of left ventricular hypertrophy, and the severity of intrarenal arteriolosclerosis, as previously described (22, 24).

**Histopathology.** Sections were cut at 4 μm and stained with periodic acid-Schiff (PAS)-hematoxylin or picrosirius red. The percentage of glomerulosclerosis and proportion of arterial intimal thickening were assessed using PAS-hematoxylin-stained sections. The percentage of sclerotic glomeruli was estimated by counting sclerosed and nonsclerosed glomeruli in 100 nonoverlapping microscopic fields from the subcapsular surface to the inner cortex with at least 400 sampled glomerular cross sections/subject. The severity of arteriosclerosis was assessed by the intimal thickness ratio, which was calculated as the proportion of the thickness of the intima and outer wall diameter at a magnification of ×400 in interlobular arteries with a diameter between 90 and 250 μm using the linear measurement function of Image-Pro Plus morphometric software (Media Cybernetics, Bethesda, MD). Cortical fibrosis was measured in ×200 images as the proportion of cortex that stained red with picrosirius stain using the automated Image-Pro Plus area counting function.

**Estimation of total nephron number.** Design-based stereology was performed at Monash University. Nephron number was estimated as previously described in detail (6, 20, 22) applying a combination of systematic uniform random sampling and the physical disector/fractionator approach.

**Estimation of glomerular volume and cell numbers.** We estimated individual glomerular volumes (IGV) and total numbers of podocytes per glomerulus using design-based stereology as previously described (49). This method is considered the gold standard for the quantification of podocyte number and glomerular volume when sufficient tissue is available (35). In brief, 50 serial paraffin sections at 14 μm thickness were obtained for each kidney. Using these sections, 12 glomeruli (4 glomeruli each from the outer, middle, and inner cortex) per subject were sampled using physical dissectors (52, 54). Section profiles of these 12 sampled glomeruli were then imaged using an Olympus DotSlide system, generally providing between 6 and 12 profiles/glomerulus. Glomerular profiles were labeled with a flag and a unique identifier. These virtual images served as maps to find profiles during confocal microscopy. From 17 of 19 subjects, 6 glomeruli were randomly selected (first 6 glomeruli that were observed) from the 12 sampled glomeruli for the estimation of glomerular volume using the Cavalieri estimator (17) and subsequent estimation of glomerular cell numbers. Data for the remaining two subjects were reported in a previous publication (48) with a different aim and analysis.

Every second section was then immunostained to facilitate cell identification and counting. Sections were immunostained using an antibody against Wilms’ tumor (WT)-1 antigen [monoclonal mouse anti-human WT-1, M356101, DAKO, clone 6F-H2 (1:50), for podocyte identification] and an antibody against von Willebrand factor (vWF; 1:200), which in this case was polyclonal rabbit anti-human vWF (A008202, DAKO, for endothelial cell identification). As in our previous study on this autopsy series (48), WT-1 immunostaining was found exclusively in the podocyte cytoplasm (Fig. 1). While 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 (41). The present localization of WT-1 immunostaining in the podocyte cytoplasm confirms the findings of Su et al. (60), who used this same antibody and reported

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**Fig. 1.** Identification of glomerular podocytes using Wilms’ tumor (WT)-1. **A:** three-dimensional reconstruction of WT-1-positive cells (Imaris 7.2, Bitplane) showing characteristic the podocyte phenotype. **B–D:** representative confocal images of a glomerulus stained for synaptopodin (SNP) and 4’,6-diamidino-2-phenylindole (DAPI; **B**) and WT-1 (**C**) as well as a merged image (**D**). Boxes in **D** are expanded in **E** and show the expression of both WT-1 and SNP in the podocyte cytoplasm.
immunostaining of the human podocyte cytoplasm, agreeing with the manufacturer’s (DAKO) specifications. Validation of WT-1 cytoplasmic expression in human podocytes has been previously described in greater detail in a recent publication (48).

Podocytes were counted in a total of 114 glomeruli (6 glomeruli from each of the 19 subjects). Every immunostained section from each of the six subsampled glomeruli per subject was imaged with a Leica SP5 laser confocal microscope (Leica Microsystems, Mannheim, Germany). Optical dissectors were used to sample and thereby count cells in 8 µm of the 14 µm available for each glomerular profile (see details in Ref. 49). 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 (9) macro. After all newly appearing nuclei had been identified, we defined podocytes as tuft cells that were WT-1 positive and vWF negative. Podocyte density was calculated by dividing total podocyte number per glomerulus by the volume (IGV) of that same glomerulus.

Data analysis was conducted using two approaches. A first analysis of podocyte depletion was conducted using subject as the unit by averaging data for six glomeruli per subject. A second analysis of podocyte depletion was conducted using glomeruli (rather than subject) as the unit. The analysis of glomerular distributions within groups defined by older age and hypertensive status confirmed the subject analysis above and provided additional insights into the focal nature of podocyte depletion. Each subject contributed an equal number (six) of randomly sampled glomeruli to this analysis.

Definition of parietal podocytes. Parietal podocytes were identified based on their location on Bowman’s capsule and their expression of WT-1. We reviewed every confocal image from all 114 glomeruli and categorized glomeruli into those that contained at least one parietal podocyte and those that did not contain any parietal podocytes. In this way, the proportions of glomeruli with/without parietal podocytes were obtained. It is worth noting that our glomerular sampling strategy allowed us to examine ~50% of the volume of each glomerulus (i.e., every second 14-µm paraffin section was immunostained and imaged).

Statistical analysis. Data were analyzed using GraphPad Prism (version 5.04) for Windows (La Jolla, CA) and StataCorp 2013 (Stata Statistical Software, release 13, College Station, TX). Values are expressed as means ± SD unless otherwise stated. Unpaired Student’s t-tests were used to compare two variables; F-tests for ANOVA and one-way ANOVAs with Holm-Sidak’s adjustment for multiple comparisons were used to compare three or more variables. The analysis of proportions was conducted using a proportion test calculator. Spearman rank coefficients and linear regression analyses were also performed. P values of <0.05 were considered statistically significant.

RESULTS

Demographics and histopathology. Table 1 shows the general demographics of the 19 subjects. Overall, the mean age was 42 yr (SD: 16.8 yr) with a mean body mass index of 24.8 kg/m² (SD: 6.07 kg/m²). Eight of the nineteen subjects (42%) were classified as hypertensive. Four subjects (21%) died of a cardiovascular-related cause, and eight subjects (42%) died as a result of misadventures, including accidents and homicides. Table 2 shows details on histopathology, including total nephron number, percentage of glomerulosclerosis, and proportion of cortical fibrosis and arteriosclerosis for each subject.

Young adults (n = 7, mean age: 23 yr, SD: 3.3 yr) had a lower body surface area (P < 0.05) than older subjects (n = 12, mean age: 53 yr, SD: 10.4 yr). While none of the young adults had hypertension, 8 of 12 older adults (67%) were classified as hypertensive. Furthermore, older adults had higher levels of glomerulosclerosis (P < 0.01), cortical fibrosis (P < 0.05), and arteriosclerosis (P < 0.001). Hypertensive subjects (n = 8) were significantly older (mean age: 55.4 yr, SD: 11.2 yr) than normotensive subjects (n = 11, mean age: 32.2 yr, SD: 13.1 yr, P < 0.01). Hypertensive subjects also showed higher levels of glomerulosclerosis (P < 0.001), cortical fibrosis (P < 0.01), and arteriosclerosis (P < 0.01) than normotensive subjects (Table 3). Medical records showed that five of eight hypertensive subjects received antihypertensive medication (angiotensin-converting enzyme inhibitors, β-blockers, and/or thiazides). Unfortunately, we do not have details on the duration or dose of these medications.

Table 1. General demographics

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age, yr</th>
<th>Body Mass Index, kg/m²</th>
<th>Body Surface Area, m²</th>
<th>Hypertensive Status</th>
<th>Cause of Death</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>21.49</td>
<td>1.79</td>
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<td>Cardiovascular</td>
</tr>
<tr>
<td>2</td>
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<td>1.85</td>
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<td>Accident</td>
</tr>
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<td>21</td>
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<td>1.56</td>
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<td>Accident</td>
</tr>
<tr>
<td>4</td>
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<td>1.97</td>
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<td>Homicide</td>
</tr>
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<td>Noncardiovascular</td>
</tr>
<tr>
<td>6</td>
<td>25</td>
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<td>1.72</td>
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<td>Cardiovascular</td>
</tr>
<tr>
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<td>18.59</td>
<td>1.60</td>
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<td>Homicide</td>
</tr>
<tr>
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<td>39</td>
<td>26.68</td>
<td>2.01</td>
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<td>Noncardiovascular</td>
</tr>
<tr>
<td>9</td>
<td>41</td>
<td>23.00</td>
<td>1.80</td>
<td>Normotensive</td>
<td>Noncardiovascular</td>
</tr>
<tr>
<td>10</td>
<td>43</td>
<td>18.19</td>
<td>1.57</td>
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<td>Noncardiovascular</td>
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<tr>
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<td>Unexplained</td>
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<td>Accident</td>
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<td>13</td>
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<td>1.77</td>
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<td>Noncardiovascular</td>
</tr>
<tr>
<td>14</td>
<td>53</td>
<td>22.60</td>
<td>2.07</td>
<td>Hypertensive</td>
<td>Accident</td>
</tr>
<tr>
<td>15</td>
<td>57</td>
<td>18.39</td>
<td>1.70</td>
<td>Hypertensive</td>
<td>Noncardiovascular</td>
</tr>
<tr>
<td>16</td>
<td>59</td>
<td>26.78</td>
<td>2.01</td>
<td>Normotensive</td>
<td>Cardiovascular</td>
</tr>
<tr>
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<td>2.20</td>
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<td>Cardiovascular</td>
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<tr>
<td>18</td>
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<td>Accident</td>
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<td>19</td>
<td>74</td>
<td>30.00</td>
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<td>Noncardiovascular</td>
</tr>
<tr>
<td>Mean</td>
<td>41.95</td>
<td>24.84</td>
<td>1.90</td>
<td>8/19 hypertensive</td>
<td>4/19 cardiovascular-related deaths (21%)</td>
</tr>
<tr>
<td>SD</td>
<td>16.80</td>
<td>6.07</td>
<td>0.29</td>
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</table>

Hypertensive status was categorized as normotensive or hypertensive. Cardiovascular causes of death included cardiac causes with/without coronary artery disease; noncardiovascular causes included infections, hematological disorders, neoplasias, and pulmonary embolisms.
Table 2. Histopathology analysis

<table>
<thead>
<tr>
<th>Subject</th>
<th>Nephron Number, million</th>
<th>Glomerulosclerosis, %</th>
<th>Cortical Fibrosis, proportion</th>
<th>Arteriosclerosis, proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.64</td>
<td>0.60</td>
<td>0.03</td>
<td>0.00</td>
</tr>
<tr>
<td>2</td>
<td>1.03</td>
<td>0.72</td>
<td>0.00</td>
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<td>1.60</td>
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<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>4</td>
<td>1.42</td>
<td>0.95</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>5</td>
<td>0.69</td>
<td>0.00</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>6</td>
<td>0.95</td>
<td>0.63</td>
<td>0.01</td>
<td>0.00</td>
</tr>
<tr>
<td>7</td>
<td>0.25</td>
<td>0.78</td>
<td>0.25</td>
<td>0.00</td>
</tr>
<tr>
<td>8</td>
<td>0.55</td>
<td>1.62</td>
<td>0.05</td>
<td>0.08</td>
</tr>
<tr>
<td>9</td>
<td>0.83</td>
<td>1.45</td>
<td>0.01</td>
<td>0.08</td>
</tr>
<tr>
<td>10</td>
<td>1.67</td>
<td>1.57</td>
<td>0.03</td>
<td>0.14</td>
</tr>
<tr>
<td>11</td>
<td>1.22</td>
<td>1.43</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
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<td>1.15</td>
<td>1.03</td>
<td>0.01</td>
<td>0.09</td>
</tr>
<tr>
<td>13</td>
<td>0.61</td>
<td>1.83</td>
<td>0.04</td>
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<td>0.58</td>
<td>2.79</td>
<td>0.03</td>
<td>0.05</td>
</tr>
<tr>
<td>16</td>
<td>1.30</td>
<td>1.32</td>
<td>0.04</td>
<td>0.12</td>
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<td>17</td>
<td>0.78</td>
<td>6.10</td>
<td>0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>18</td>
<td>0.56</td>
<td>6.22</td>
<td>0.01</td>
<td>0.16</td>
</tr>
<tr>
<td>19</td>
<td>0.83</td>
<td>3.61</td>
<td>0.10</td>
<td>0.14</td>
</tr>
<tr>
<td>Mean</td>
<td>0.90</td>
<td>1.92</td>
<td>0.03</td>
<td>0.07</td>
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<tr>
<td>SD</td>
<td>0.39</td>
<td>1.72</td>
<td>0.02</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Nephron number comprises the total number of nephrons in the right kidney. Glomerulosclerosis was based on the glomerulosclerotic index (in %). Cortical fibrosis is the proportion of the fibrotic cortex. Arteriosclerosis was based on the ratio of intimal thickness to outer wall diameter.

Given the clear overlap between older age and hypertension, we generated more detailed categories for further analysis. Table 4 shows demographic and histopathological data for young normotensive (n = 6), older normotensive (n = 5), and older hypertensive (n = 8) subjects. These categories allowed us to isolate the independent and additive contributions of age and hypertension. In this context, increases in arteriosclerosis were closely linked to older age (P < 0.05), increases in glomerulosclerosis were associated with hypertension (P < 0.05) rather than older age, and the combination of older age and hypertension enhanced the levels of glomerulosclerosis (P < 0.01), cortical fibrosis (P < 0.05), and arteriosclerosis (P < 0.0001).

Definition of podocyte depletion. Podocyte depletion was assessed by three main parameters: IGV (glomerular hypertrophy), total podocyte number per glomerulus (absolute depletion), and podocyte density per glomerulus (relative depletion). Values for all three parameters were estimated using design-based stereology (48, 49). Representative images of immunostained podocytes are shown in Fig. 1, including a three-dimensional reconstruction of a stack of confocal z-series optical sections showing the characteristic podocyte phenotype (Fig. 1A) based on WT-1 immunostaining. Niksic et al. (41) confirmed that WT-1 is expressed in the nucleus and cytoplasm of multiple cell types, including mouse mesonephros. Furthermore, a recent publication by Su et al. (60) showed cytoplasmic labeling of podocytes, which is consistent with previous publications by our group (48, 49). However, because of the unusual location of WT-1 immunostaining (cytoplasmic, not nuclear), we also provide representative confocal optical images of a normal glomerulus (Fig. 1, B–D) showing the expression of both WT-1 and synaptopodin in podocyte cytoplasm (Fig. 1E).

Our approach for quantifying podocyte depletion has a major advantage in that it allows the analysis of podocyte depletion on a per glomerulus basis, and, in this context, further analysis focused on each glomerulus as a unit can provide valuable insights. In this context, IGV estimates ranged from 0.76 to 8.13 × 10^6 μm^3 (10.7-fold) among the 114 glomeruli, with a much smaller range within subjects (between 1.2- and 3.3-fold). In contrast, podocyte number per glomerulus was less variable, with values ranging from 140 to 780 podocytes, a 5.6-fold range. Within subjects, the range in podocyte number per glomerulus was between 1.4- and 2.6-fold. A 7.3-fold range in podocyte density was found in the 114 glomeruli (from 57 to 417 podocytes per 10^6 μm^3), whereas podocyte density ranged from 1.2- to 3.6-fold within subjects.

Contributions of older age and hypertension to podocyte depletion: analysis per subject. Glomeruli in older adults were larger (P = 0.03; Fig. 2A) than those in young adults, contained 17% fewer podocytes (P = 0.03; Fig. 2B), and had a lower podocyte density (P < 0.01; Fig. 2C). Glomeruli from hypertensive subjects were 70% larger (P = 0.02; Fig. 2D) than glomeruli from normotensive subjects, contained similar numbers of podocytes (P = 0.21; Fig. 2E), and thus had lower podocyte density (P = 0.02; Fig. 2F).

Next, subjects were classified into three groups (young normotensive, older normotensive, and older hypertensive subjects) to assess the independent contributions of older age and hypertension to podocyte depletion. Neither older age nor hypertension had significant independent effects on glomerular size (Fig. 2G). Older age was independently associated with...
lower podocyte number ($P = 0.01$; Fig. 2H) and lower podocyte density ($P = 0.04$; Fig. 2I). In contrast, hypertension was not independently associated with reductions in podocyte number or density. The combination of older age and hypertension was associated with increases in glomerular volume ($P = 0.04$) and decreases in podocyte number ($P = 0.02$) and density ($P = 0.01$).

**Contributions of older age and hypertension to podocyte depletion: analysis per glomerulus.** Glomeruli from older adults were larger ($P < 0.0001$; Fig. 3A) and more variable in size ($P < 0.0001$) than glomeruli from young adults. As shown in Fig. 3B, glomeruli from older adults contained fewer podocytes ($P < 0.01$) than glomeruli from young adults but showed similar variability in terms of podocyte number ($P = 0.85$). Figure 3C shows that glomeruli from older subjects had a much lower podocyte density ($P < 0.0001$) than glomeruli from young adults but similar variance ($P = 0.48$).

Glomeruli from hypertensive subjects were larger ($P < 0.0001$; Fig. 3D) and more variable in size ($P < 0.0001$) than glomeruli from normotensive subjects. As shown in Fig. 3E, glomeruli from hypertensive adults contained a similar number of podocytes ($P = 0.05$) with a similar distribution ($P = 0.64$) as glomeruli from normotensive adults. Figure 3F shows that glomeruli from hypertensive subjects had a much lower podocyte density ($P < 0.0001$) than glomeruli from normotensive adults but similar variance ($P = 0.68$).

Next, the independent contributions of older age and hypertension to podocyte depletion were also assessed. Figure 3G shows that hypertension ($P < 0.0001$), but not older age ($P = 0.21$), was an independent contributor to increases in glomerular volume. In contrast, age ($P < 0.01$), but not hypertension ($P = 0.76$), was an independent contributor to decreases in podocyte number (Fig. 3H). Figure 3I shows that both older age ($P < 0.0001$) and hypertension ($P < 0.01$) are independent contributors to reductions in podocyte density. Not surprisingly, the combination of older age and hypertension was associated with increases in glomerular size ($P < 0.0001$) and decreases in podocyte number ($P < 0.01$) and podocyte density ($P < 0.0001$).

**Associations between podocyte number and glomerular size in the context of age and hypertension.** As shown in Fig. 4A, podocyte number was directly associated with glomerular volume in both young ($R = 0.58$, $P < 0.0001$) and older ($R = 0.29$, $P < 0.05$) adults but with clearly different slopes ($F$: 6.4, $P = 0.01$); in young subjects, each additional $1 \times 10^5$ μm$^3$ of glomerular volume was associated with 93 additional podocytes ($R^2 = 0.22$, $P < 0.01$), whereas in older subjects, this same increase in glomerular size was only associated with an additional 15 podocytes ($R^2 = 0.06$, $P < 0.05$). Analysis of individual glomerular volumes by tertiles showed that glomeruli from older adults were significantly larger than glomeruli from young adults at every level of glomerular size ($P < 0.0001$; Fig. 4B). While age-related differences in podocyte number were only observed in the largest glomeruli ($P < 0.01$; Fig. 4C), decreases in podocyte density were present at every level of glomerular size ($P < 0.0001$; Fig. 4D).

Figure 4E shows that podocyte number was weakly associated with glomerular volume in both normotensive ($R = 0.27$, $P < 0.05$) and hypertensive ($R = 0.29$, $P < 0.05$) subjects and had similar slopes ($F$: 0.07, $P = 0.79$). Tertile analysis revealed that glomeruli from hypertensive subjects were significantly larger than glomeruli from normotensive adults at every level of glomerular size ($P < 0.01$ for small glomeruli and $P < 0.0001$ for medium and large glomeruli; Fig. 4F). Tertile analysis also confirmed no differences in podocyte number between normotensive and hypertensive subjects ($P > 0.05$ at every tertile of glomerular size; Fig. 4G). Furthermore, hypertensive subjects had lower podocyte density than normotensive subjects at every tertile of glomerular size ($P < 0.0001$ for small glomeruli and $P < 0.0001$ for medium and large glomeruli; Fig. 4H).

The results shown in Fig. 4I confirm the strong association between podocyte number and glomerular volume in young normotensives ($R = 0.48$, $P < 0.01$), with weaker associations present in older normotensive subjects ($R = 0.36$, $P < 0.05$) and older hypertensive subjects ($R = 0.29$, $P < 0.05$). Importantly, the association between IGV and podocyte number was remarkably similar in older normotensive and hypertensive subjects, suggesting that reductions in podocyte number are mostly determined by aging rather than hypertension ($F$: 0.17, $P = 0.69$). However, the range of IGV in older hypertensive subjects was twice that found in older normotensive subjects, marking an additional level of glomerular hypertrophy when older age and hypertension are present in combination. Tertile analysis revealed that the independent contributions of older age and hypertension to podocyte depletion indexes were
mostly present among medium and large glomeruli (Fig. 4, J–L). These analyses confirmed findings from the analysis by subject presented above, namely, that age is independently associated with increases in glomerular volume ($P < 0.01$), reductions in podocyte number ($P < 0.05$), and podocyte density ($P < 0.001$ for medium glomeruli and $P < 0.0001$ for large glomeruli), whereas hypertension is independently associated with increases in glomerular volume ($P < 0.0001$) and decreases in podocyte density ($P < 0.001$ in large glomeruli only) but has no association with podocyte number ($P > 0.05$).

**Cortical zones and podocyte depletion.** Overall, there were no differences in IGV, podocyte number, or podocyte density between glomeruli located in the outer and inner cortex ($P > 0.05$ for all variables).

Outer glomeruli from older adults tended to be larger ($P = 0.07$; Fig. 5A), contained 23% fewer podocytes ($P < 0.05$; Fig. 5B), and showed a 60% reduction in podocyte density ($P < 0.001$; Fig. 5C) compared with glomeruli from young adults. In contrast, none of these differences were observed in association with hypertension.

Glomeruli from the inner cortex were significantly larger in association with both older age (62%, $P < 0.01$) and hypertension (65%, $P < 0.0001$; Fig. 5D). These inner glomeruli contained 24% fewer podocytes ($P < 0.01$) in association with older age and tended to contain fewer podocytes (15%, $P = 0.06$) in association with hypertension (Fig. 5E). Consequently, podocyte density in inner glomeruli was significantly reduced in the setting of older age and hypertension ($P < 0.0001$ in both cases; Fig. 5F).

Older age was independently associated with increased glomerular volume ($P < 0.05$ for both the outer and inner cortex; Fig. 5G), a tendency for lower numbers of podocytes in the outer cortex ($P = 0.08$), and reduced podocyte number in the inner cortex ($P < 0.05$; Fig. 5H). Podocyte density was also
significantly reduced (P < 0.01 for the outer cortex and P < 0.001 for the inner cortex; Fig. 5I). Remarkably, hypertension had no effect on these parameters for glomeruli in the outer cortex. However, hypertension was associated with glomerular hypertrophy (P < 0.01) and reduced podocyte density (P < 0.05) in glomeruli from the inner cortex.

**Presence of WT-1-positive parietal epithelial cells.** Parietal podocytes can be defined as parietal epithelial cells (PECs) that express podocyte markers; in this case, WT-1-positive PECs (Fig. 6A). Parietal podocytes were observed in 87 of 114 glomeruli (76%) studied and were mostly located near the vascular pole. Figure 6B shows that the proportion of glomeruli with parietal podocytes increased with age (P < 0.001) but not with hypertension (P > 0.05). Furthermore, Fig. 6C shows that older age, unlike hypertension, was independently associated with an increase in the proportion of glomeruli with parietal podocytes (P < 0.01).

**DISCUSSION**

The major finding of this study is that older age and hypertension are independent contributors to podocyte depletion in subjects without renal disease. Older age was associated with absolute and relative podocyte depletion, whereas hypertension was only associated with relative podocyte depletion. Podocyte depletion was most marked when both older age and hypertension were present.

To date, only a handful of studies have estimated the total number of podocytes in adult human glomeruli, and these studies primarily focused on glomerular disease. Studies of type 2 diabetes identified absolute podocyte depletion in the early stages of diabetic nephropathy, which was closely related to disease progression (11, 38, 47, 60). Absolute podocyte depletion was also reported in patients with IgA nephropathy and hypertensive nephrosclerosis among patients with CKD.
These previous studies provided important insights into the association between podocyte depletion and the onset and progression of renal disease. However, a key finding in the present study is of lower podocyte number and density in subjects with older age and hypertension but without overt disease. This study thus provides unique insights into the degrees of podocyte depletion that can exist in the human kidney before the onset of overt pathology.

In 2005, Wharram et al. (65) described a transgenic rat model in which the human diphtheria toxin receptor was specifically expressed in podocytes to achieve dose-dependent podocyte depletion. This study showed that death of 20% of podocytes resulted in FSGS with sustained proteinuria with normal renal function. More importantly, Wharram et al. (65) provided evidence that podocyte loss was sufficient for the development of progressive glomerular disease after a certain threshold was reached. Such information is not available in humans but may represent a significant advance in the field.

The present study adds to our understanding of absolute podocyte depletion in humans, showing that a 30-yr age difference (23.0 ± 3.3 vs. 53.0 ± 10.4 yr) was associated with a 24% reduction in podocyte number without any signs of overt glomerular disease, suggesting that this moderate level of absolute podocyte depletion is not sufficient to cause glomerular pathology.

More than 20 years ago, Nagata and Kriz (40) showed that in a setting of glomerular hypertrophy, podocytes can undergo significant hypertrophy to cover the enlarged capillary area. However, a key finding in the present study is of lower podocyte number and density in subjects with older age and hypertension but without overt disease. This study thus provides unique insights into the degrees of podocyte depletion that can exist in the human kidney before the onset of overt pathology.

In 2005, Wharram et al. (65) described a transgenic rat model in which the human diphtheria toxin receptor was specifically expressed in podocytes to achieve dose-dependent podocyte depletion. This study showed that death of >20% of podocytes resulted in FSGS with sustained proteinuria with normal renal function. More importantly, Wharram et al. (65) provided evidence that podocyte loss was sufficient for the development of progressive glomerular disease after a certain threshold was reached. Such information is not available in humans but may represent a significant advance in the field. The present study adds to our understanding of absolute podocyte depletion in humans, showing that a 30-yr age difference (23.0 ± 3.3 vs. 53.0 ± 10.4 yr) was associated with a 24% reduction in podocyte number without any signs of overt glomerular disease, suggesting that this moderate level of absolute podocyte depletion is not sufficient to cause glomerular pathology.
segmental glomerulosclerosis. However, it is worth noting that
the pattern of glomerulosclerosis occurring in association
with older age and the early stages of hypertensive nephrosclerosis
is global rather than segmental (13, 16, 24). Furthermore, a
recent publication (28) has shown the importance of identify-
ing the difference between age-related and disease-related
glomerulosclerosis. Using this tool, and after adjustments for
our sampling strategy (400 glomeruli/subject vs. up to 64
glomeruli/subject in Kremers et al. (28)), we determined that
all our subjects presented age-related glomerulosclerosis,
which suggests that these subjects did not show overt glomer-
ular disease.

More than 30 yr ago, Olivetti et al. (44) showed in rats that
the number of podocytes increased in early postnatal life in
association with glomerular hypertrophy. We recently showed
that large adult human glomeruli contain more podocytes than
glomeruli of children, supporting the possibility of podocyte
gain during periods of early body growth (48). Nevertheless,
this apparent increase in podocyte number (~20%) is far less
than the 700% increase in glomerular volume that occurs
between childhood and adulthood, thereby leading to a reduc-
tion in podocyte density per glomerulus. Our present findings
highlight that among young adults, large glomeruli contain
more podocytes than small glomeruli, which confirms our
previous publication (48) and keeps the following two main
hypotheses alive:

1) glomeruli with a high podocyte endow-
ment (podocyte number at birth) are capable of undergoing
significant glomerular hypertrophy and/or
2) glomerular hy-
pertrophy is associated with potential increases in podocyte
density per glomerulus. In this context, it is worth exploring
the possible contribution of PECs toward podocyte turnover.

Bariety et al. (2) described the presence of parietal podo-
cytes in the normal human kidney and reported the presence
of parietal podocytes in 76.6% of examined glomeruli. In the

Fig. 5. Cortical location and podocyte depletion. A: IGV by age categories and hypertensive status in outer glomeruli. B: total podocyte number per glomerulus by age categories and hypertensive status in outer glomeruli. C: podocyte density per glomerulus by age categories and hypertensive status in outer glomeruli. D: IGV by age categories and hypertensive status in inner glomeruli. E: total podocyte number per glomerulus by age categories and hypertensive status in inner glomeruli. F: podocyte density per glomerulus by age categories and hypertensive status in inner glomeruli. G: IGV by age and hypertension in outer and inner glomeruli. H: total podocyte number per glomerulus by age and hypertension in outer and inner glomeruli. I: podocyte density per glomerulus by age and hypertension in outer and inner glomeruli. Each circle represents one glomerulus. Error bars represent means ± SD (lines or squares). NT, normotensive subjects; HT, hypertensive subjects.
present study, 76% of glomeruli contained parietal podocytes, and the proportion of glomeruli with parietal podocytes was increased in association with older age, which may require podocyte gain. Interestingly, Appel et al. (1) demonstrated that parietal podocytes in the young mouse kidney were able to migrate from Bowman’s capsule to the glomerular tuft. While Wanner et al. (63) ruled out podocyte turnover by PEC differentiation/migration during adulthood, Berger et al. (3, 5) postulated that this small capacity to gain podocytes is mostly present during periods of body growth (childhood/adolescence) and that it is somehow lost in the adult mouse kidney. Recent studies have also supported the possibility of PECs as podocyte progenitors (14, 33). Given the cross-sectional nature of our study, it is impossible to define if these parietal cells belong to 1) a podocyte reservoir, which means they were present from birth (3), or 2) are part of an active progenitor pool, meaning they have differentiated into parietal podocytes from multipotent progenitors (57). We postulate that parietal podocytes may play a role in podocyte gain throughout life to keep podocyte number/density within a healthy physiological range. However, older age and hypertension are associated with podocyte depletion, which suggests that podocyte loss and glomerular hypertrophy overpower any possible mechanism of podocyte gain.

Steffes et al. (58) reported no difference in podocyte number between subjects aged younger and older than 20 yr, which supported the hypothesis that numbers of podocytes remain constant across the human lifespan. However, Hodgin et al. (19) showed that aging is closely associated with a reduction in human podocyte number and, more importantly, podocyte density, which, in turn, causes podocyte hypertrophy that can result in glomerular tuft collapse and glomerulosclerosis. The present study supports these findings and adds additional details in relation to the contribution of hypertension and the role of zonal location.

Animal studies have suggested that the main mechanism for podocyte loss is podocyte detachment from the glomerular basement membrane (32), a process that may be exacerbated by hypertension (31). Furthermore, evidence suggests that increases in intraglomerular pressure may contribute to glomerular hypertrophy and thereby relative podocyte depletion. The findings of Wang et al. (62) support this theory, showing absolute and relative podocyte depletion in adult patients with hypertensive nephrosclerosis within the context of moderate CKD. While the present study shows that hypertension is associated with glomerular hypertrophy and thereby reductions in podocyte density, we did not observe a difference in total podocyte number solely driven by hypertension. However, a limitation of the present study is the lack of young hypertensive subjects, which would have allowed us to further examine the contribution of hypertension to podocyte depletion in younger individuals.

Samuel et al. (54) proposed that zonal location could be an important determinant of glomerular hypertrophy in the human kidney. Furthermore, a previous publication by our group showed that juxtamedullary glomeruli are usually larger than superficial glomeruli under normal conditions, but this difference is lost in subjects with CKD risk factors such as older age, hypertensive nephrosclerosis.
hypothesis, and obesity (52). At first, we found no differences in glomerular size, podocyte number, or podocyte density by cortical location. However, a more detailed analysis revealed that age-related podocyte depletion affected glomeruli across the entire renal cortex, being more pronounced in glomeruli located in the inner cortex. These findings are supported by a recent animal study (53) that described exacerbated age-related podocyte depletion and PEC activation in juxtamedullary glomeruli of old mice. Moreover, the present study shows that hypertension-related relative podocyte depletion is predominantly observed in juxtamedullary glomeruli, affecting glomeruli located in the proximity of the arcuate arteries, which may be more susceptible to elevations in systemic and glomerular pressure. Overall, our findings highlight the importance of cortical zone as a determinant of podocyte depletion in humans and raise an interesting question into the field of diagnostic pathology: given that renal biopsies rarely contain juxtamedullary glomeruli, are we underestimating the degree of podocyte depletion/injury? This question will remain unanswered until noninvasive assessment of podocyte depletion becomes a reality.

The present study has several limitations. We acknowledge the inherent bias of cross-sectional studies, particularly in a very specific cohort, such as male Caucasian Americans. An important caveat is the difficulty to extrapolate these findings to women and other racial groups. While we have knowledge of antihypertensive medication prescriptions in the medical records, we do not know the duration, dose, or effectiveness of these drugs, which may have an impact on our findings. We emphasize that although the number of subjects is small (19 subjects), the detail, precision, and accuracy of our quantitative analysis in a large sample of glomeruli (n = 114 glomeruli) is significant, especially when considering this is precious human autopsy tissue and the challenges of applying design-based stereology. In the present study, we analyzed podocyte depletion using both subjects and glomeruli as units. The findings from the latter analysis confirmed and extended the findings from the subject analysis. It can be argued that glomeruli within an individual are not independent from each other, which means that the analysis of glomeruli as a unit may be confounded. However, the great variability in glomerular size, podocyte number, and podocyte density within subjects suggest that despite being exposed to similar stressors, the effect of this stimulus may differ between glomeruli. We believe that the analysis of glomerular distributions within each category provides important insights and is relevant to understanding the focal nature of glomerular disease.

In conclusion, aging and hypertension are independent contributors to podocyte depletion in human kidneys without overt renal disease. Furthermore, the combination of both older age and hypertension accentuates the degree of podocyte depletion and may render glomeruli more susceptible to glomerulosclerosis. Further studies are needed to validate these findings in larger cohorts.

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HUMAN PODOCYTE DEPLETION WITH OLDER AGE AND HYPERTENSION


